

กลไกของสารสกัดสมุนไพรไทยในการออกฤทธิ์ด้านความเป็นพิษของกลูตาเมตต่อเซลล์ประสาท



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บทคัดย่อและแฟ้มข้อมูลฉบับเต็มของวิทยานิพนธ์ตั้งแต่ปีการศึกษา 2554 ที่ให้บริการในคลังปัญญาจุฬาฯ (CUIR)

เป็นแฟ้มข้อมูลของนิสิตเจ้าของวิทยานิพนธ์ ที่ส่งผ่านทางบัณฑิตวิทยาลัย

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สาขาวิชาชีวเคมีคลินิกและอณูทางการแพทย์ ภาควิชาเคมีคลินิก

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ปีการศึกษา 2560

ลิขสิทธิ์ของจุฬาลงกรณ์มหาวิทยาลัย



MECHANISMS OF THAI MEDICINAL PLANT EXTRACTS ON THE ATTENUATION OF GLUTAMATE-MEDIATED NEUROTOXICITY

Miss Anchalee Prasansuklab



A Dissertation Submitted in Partial Fulfillment of the Requirements  
for the Degree of Doctor of Philosophy Program in Clinical Biochemistry and

Molecular Medicine

Department of Clinical Chemistry

Faculty of Allied Health Sciences

Chulalongkorn University

Academic Year 2017

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Thesis Title	MECHANISMS OF THAI MEDICINAL PLANT EXTRACTS ON THE ATTENUATION OF GLUTAMATE-MEDIATED NEUROTOXICITY
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Field of Study	Clinical Biochemistry and Molecular Medicine
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ัญชลี ประสารสุขาลม : กลไกของสารสกัดสมุนไพรไทยในการออกฤทธิ์ด้านความเป็นพิษของกลูตาเมตต่อเซลล์ประสาท (MECHANISMS OF THAI MEDICINAL PLANT EXTRACTS ON THE ATTENUATION OF GLUTAMATE-MEDIATED NEUROTOXICITY) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ผศ. ดร.เทวิน เทนคำเนาว์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ. ดร. Alison T Ung, 210 หน้า.

อัตราการเกิดของโรคในกลุ่มโรคที่เกิดความเสื่อมทางระบบประสาท โดยเฉพาะโรคอัลไซเมอร์ มีแนวโน้มที่เพิ่มสูงขึ้นเรื่อยๆ ซึ่งปัจจัยหนึ่งนั้นมาจากการเพิ่มจำนวนของประชากรสูงอายุทั่วโลก อย่างไรก็ตาม ปัจจุบันยังไม่มีวิธีการรักษาให้หายขาดจากโรคในกลุ่มนี้ได้ การรักษาด้วยสมุนไพรอาจเป็นหนึ่งในทางเลือกที่มีประสิทธิภาพสำหรับโรคที่เกิดจากความเสื่อมของระบบประสาท ซึ่งประเทศไทยนั้นอุดมไปด้วยทรัพยากรธรรมชาติ รวมถึงพืชสมุนไพรนานาชนิดที่ยังไม่ได้รับการศึกษาถึงประโยชน์อย่างชัดเจน รายงานก่อนหน้านี้พบว่าความผิดปกติของระบบสารสื่อประสาทกลูตาเมตเกี่ยวข้องกับการเกิดพยาธิสภาพของโรค โดยภาวะที่ปริมาณกลูตาเมตภายนอกเซลล์สูงมากผิดปกตินั้น สามารถนำไปสู่การตายของเซลล์ประสาทได้ ดังนั้นการศึกษาในครั้งนี้จึงมีวัตถุประสงค์เพื่อศึกษาฤทธิ์และกลไกการออกฤทธิ์ของสารสกัดหยาบจากสมุนไพรในการปกป้องการตายของเซลล์ประสาทจากภาวะเครียดออกซิเดชันที่ถูกเหนี่ยวนำด้วยกลูตาเมต ซึ่งภาวะดังกล่าวเป็นปัจจัยสำคัญต่อการลดจำนวนลงของเซลล์ประสาท รวมถึงเพื่อประเมินฤทธิ์ด้านความชราของสารสกัดด้วย โดยคณะผู้วิจัยได้เลือกศึกษาพืชสมุนไพรไทยจำนวน 5 ชนิด คือ ยอ ผักปวยล่า หนำปึกกิ่ง เหงือกปลาหมอ และข่อย และได้ทำการประเมินฤทธิ์ของสารสกัดหยาบจากพืชสมุนไพรไทยในการต้านภาวะเครียดออกซิเดชันและปกป้องการตายของเซลล์จากพิษกลูตาเมต ด้วยเทคนิคการตรวจวัดความมีชีวิตของเซลล์ การตายของเซลล์แบบอะพอพโทซิส และระดับของอนุมูลอิสระ ในเซลล์ประสาทชนิด HT-22 ซึ่งเป็นเซลล์แบบจำลองสำหรับการทดสอบภาวะความเป็นพิษกลูตาเมต อีกทั้งยังได้ทำการศึกษากลไกการออกฤทธิ์โดยการวิเคราะห์การแสดงออกของยีนที่สนใจในระดับอาร์เอ็นเอและโปรตีนด้วยการทดสอบอิมมูโนฟลูออเรสเซนซ์ เทคนิคเวสเทิร์นบลอต และเทคนิคการทำปฏิกิริยาลูกโซ่พอลิเมอเรสเรียลไทม์แบบถอดรหัสย้อนกลับ ทำการประเมินฤทธิ์ด้านความชราด้วยการทดสอบอายุขัยของหนอนพยาธิตัวกลม *C. elegans* รวมถึงทำการวิเคราะห์สารพิษและสารสกัดแยกย่อยสารสกัดหยาบโดยใช้กรดหรือเบส เพื่อตรวจสอบถึงคุณลักษณะของสารออกฤทธิ์สำคัญในพืชสมุนไพร ผลการศึกษาพบว่าเหงือกปลาหมอและข่อยสามารถออกฤทธิ์ได้ดีที่สุดจากสมุนไพรทั้งหมด 5 ชนิด โดยสารสกัดหยาบเอทานอลจากใบเหงือกปลาหมอและข่อยสามารถลดการตายของเซลล์ประสาทจากพิษกลูตาเมตได้ตามความเข้มข้นของสารที่เพิ่มขึ้นอย่างมีนัยสำคัญทางสถิติ โดยพบว่าสารสกัดนั้นไปยับยั้งทั้งการตายของเซลล์แบบอะพอพโทซิสซึ่งถูกชักนำโดยตัวกระตุ้นอะพอพโทซิส AIF นอกจากนี้ สารสกัดทั้งสองยังสามารถไปลดการเกิดอนุมูลอิสระภายในเซลล์ที่เกิดจากการเหนี่ยวนำของกลูตาเมต และยังเพิ่มการแสดงออกในระดับอาร์เอ็นเอและโปรตีนของยีนทรานสคริปชันแฟกเตอร์ Nrf2 รวมถึงยีนต้านอนุมูลอิสระต่างๆ ที่อยู่ภายใต้การควบคุมของ Nrf2 ได้อย่างมีนัยสำคัญทางสถิติ อีกทั้งสารสกัดจากใบข่อยยังสามารถเพิ่มอายุขัยของหนอนพยาธิ *C. elegans* ได้ด้วย คณะผู้วิจัยพบว่าสารพิษและสารสำคัญที่คาดว่าจะออกฤทธิ์ปกป้องเซลล์ในการศึกษาครั้งนี้คือสาร verbascoside ในใบเหงือกปลาหมอและสาร carnosic acid ในใบข่อย อย่างไรก็ตามเมื่อทำการสกัดแยกย่อยสารสกัดหยาบเอทานอลจากใบข่อยตามความเป็นกลาง กรด และเบสของสารพิษและสารสำคัญ พบว่าสารสกัดย่อยที่เป็นกลางสามารถออกฤทธิ์ในการปกป้องเซลล์จากพิษกลูตาเมตได้ดีที่สุด ในขณะที่สารสกัดย่อยที่เป็นกรดสามารถออกฤทธิ์ต้านอนุมูลอิสระได้ดีที่สุด นอกจากนี้ยังพบว่าในสารสกัดย่อยที่เป็นกลางและกรดมีสารพิษและสารสำคัญอย่างน้อย 1 ชนิดที่มีฤทธิ์ด้านเอนไซม์อะเซทิลโคลีนเอสเตอเรสได้ จากผลการศึกษาทั้งหมดคณะผู้วิจัยสรุปว่า สารสกัดหยาบเอทานอลจากใบเหงือกปลาหมอและข่อยมีฤทธิ์ทั้งในการปกป้องเซลล์ประสาทและการต้านความชรา และเป็นแหล่งของสารสำคัญซึ่งอาจนำมาพัฒนาเป็นยาทางเลือกใหม่ในการรักษากลุ่มโรคที่เกิดความเสื่อมทางระบบประสาทต่อไปได้ในอนาคต

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# # 5476953437 : MAJOR CLINICAL BIOCHEMISTRY AND MOLECULAR MEDICINE

KEYWORDS: NEURODEGENERATIVE DISEASES / ALZHEIMER'S DISEASE / GLUTAMATE / OXIDATIVE STRESS / STREBLUS ASPER / ACANTHUS EBRACTEATUS / NEUROPROTECTION

ANCHALEE PRASANSUKLAB: MECHANISMS OF THAI MEDICINAL PLANT EXTRACTS ON THE ATTENUATION OF GLUTAMATE-MEDIATED NEUROTOXICITY. ADVISOR: ASST. PROF. TEWIN TENCOMNAO, Ph.D., CO-ADVISOR: ASSOC. PROF. ALISON T UNG, Ph.D., 210 pp.

As the world population ages, neurodegenerative diseases, particularly Alzheimer's disease, are becoming a major public health concern with increasing incidence and prevalence worldwide while no cure currently exists. To challenge this situation, herbal medicine may provide a potential alternative treatment as use of natural-derived substances has been proven to be effective in the prevention and/or treatment of several diseases. During the past decades, increasing evidence has implicated excessive glutamate levels in the pathway of neuronal cell death. Thailand is a place known for cultivating a variety of tropical plants and herbs, so far many of them have never been examined for their benefits in neuroprotection. Therefore, this study aimed to investigate the effects of Thai medicinal plant extracts and their underlying mechanisms on the attenuation of glutamate-induced neuronal cell death in the mechanism of oxidative glutamate toxicity using hippocampal neuronal HT-22 cell line as *in vitro* model of neurodegeneration. We selected five Thai medicinal plant species including *Morinda citrifolia*, *Caesalpinia mimosoides*, *Murdannia loriformis*, *Acanthus ebracteatus*, and *Streblus asper*. Then the cell viability, apoptosis, radical scavenging and DCFH-DA assays were performed to assess the protective effects of plant extracts against glutamate-induced oxidative toxicity in HT-22 cells. To elucidate the underlying mechanisms, cells treated with plant extracts were analyzed for the expressions of mRNA and proteins interested by immunofluorescent staining, western blot analysis and quantitative real-time reverse transcription polymerase chain reaction techniques. The longevity effect of plant extracts was examined on *C.elegans* by lifespan assay. Phytochemical analysis and separation using Acid-Base extraction were performed to clarify putative phytochemical components of the promising extracts. Here, among five plant species, *A. ebracteatus* and *S. asper* exhibited profound neuroprotective properties. We demonstrate that the ethanol leaf extracts of both plant species are capable of attenuating the oxidative stress and HT-22 cell death induced by glutamate in a concentration-dependent manner. Co-treatment of glutamate with both extracts significantly reduced apoptotic cell death via inhibition of apoptotic-inducing factor nuclear translocation. The increases in nuclear factor erythroid 2-related factor 2 (Nrf2) levels in the nucleus and gene expression levels of antioxidant-related downstream genes under Nrf2 control were found to be significant in cells treated with both plant extracts. Furthermore, the extract of *S. asper* was able to extend the lifespan of *C.elegans*. Phytochemical analysis of chemical components proposed at least two molecules of interest include verbascoside from *A. ebracteatus* and carnolic acid from *S. asper*, which are possibly responsible for their neuroprotective properties. Further isolation of *S. asper* ethanol leaf extract into three fractions showed that the antioxidant activities were found in the order of acidic > basic > neutral, whereas the decreasing order of neuroprotective activity was neutral > basic > acidic. Moreover, TLC bioautography revealed one component in the neutral fraction exhibited anti- acetylcholinesterase (AChE) activity. While in the acid fraction, two components showed inhibitory activity against AChE. Taken together, the overall findings have supported the potential of *A. ebracteatus* and *S. asper* leaf as promising natural sources for neuroprotective agents, anti-aging agents, and/or AChE inhibitors.

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Academic Year: 2017

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## ACKNOWLEDGEMENTS

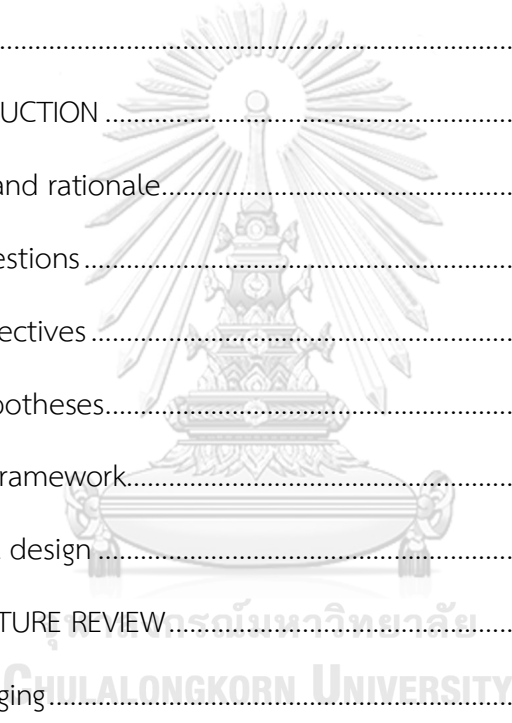
After an intensive period of my Ph.D. study, it finally comes to the day of finishing my dissertation. I would not come this far without continuous support from many people. First of all, I would like to express my deepest gratitude to my advisor, Asst. Prof. Dr. Tewin Tencomnao, who guided, supported and encouraged me throughout difficult times on my graduate study. He was always busy and tired from working, but he tried to give students his time as much as he could. He is optimistic and often finds things amusing which made me and people around him happy and relaxed. He gave me an inspiration that I will never forget and one day I wish I can be a great teacher like him. I would also like to thank my oversea co-advisor, Assoc. Prof. Dr. Alison T. Ung, for her kind supervision, guidance, support and encouragement. I am really appreciated her invaluable and willing help during the time I stayed in Sydney. She has taught me many things, not only in the scientific area, but also on a personal level for spending a good life.

I would like to thank the Chair of my dissertation committee, Assoc. Prof. Dr. Rachana Santiyanont. I am most grateful for her professional teaching and advice since my undergraduate study. I would also like to thank the other three dissertation committee members, Dr. Siriporn Sangsuthum, Dr. Tewarit Sarachana and Dr. Warunya Arunotayanun for generously offering their valuable time, ideas and suggestions to improve my work. I would like to thank a Chulalongkorn University Graduate Scholarship to commemorate the 72nd Anniversary of His Majesty King Bhumibol Adulyadej, an Overseas Research Experience Scholarship for Graduate Student by the Graduate School, Chulalongkorn University, and a Grant for Joint Funding, Ratchadaphiseksomphot Endowment Fund, for providing opportunities to carry on my Ph.D. study at Chulalongkorn University and to experience in organic chemistry research at University of Technology Sydney (UTS) for six months. Moreover, this work would not have been achieved without the financial support of the National Research University Project, Office of the Higher Education Commission (WCU-058-AS-57) and the 90th anniversary of Chulalongkorn University fund. My thanks also go to faculty of allied health sciences, Chulalongkorn University and faculty of science, UTS and Assoc. Prof. Dr. Krai Meemon and Prof. Prasert Sobhon from Faculty of Science, Mahidol University for supporting certain laboratories materials and instruments, as well as to the Princess Maha Chakri Sirindhorn Herbal Garden (Rayong Province, Thailand) for providing plant materials.

I would like to express my sincere thanks to all of my colleagues and lecturers in the department of clinical chemistry for their expertise and technical support. I am very grateful to all my friends who always support and encourage me. My special thanks go to Prof. David Schubert (The Salk Institute, USA) for his generous gift of HT-22 cells, Prapaporn Jattujan for her assistance in the C.elegans study, Varaporn Rakkhitawatthana for her help in providing plant extracts used in preliminary study, Atsadang Theerasri for his kind help in MTT experiment of fractions, Matthew Payne for his kind help in antibacterial experiment, Matthew Phillips for his willing support during my stay at UTS, and Wipada Chanakiat for her friendship and always standing by my side. Last but not least, nobody has been more important to me in the pursuit of Ph.D. study than the members of my family. I am heartily thankful to my lovely parents, younger sister and brother, who always support and encourage me throughout my entire life, and trust me in everything I do. They are always there for me, so that I can try my best in everything.



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## CHAPTER I

### INTRODUCTION

#### 1.1 Background and rationale

The achievements of public health care programs, as well as the significant progress in socio-economic development over the past century have led to declining mortality rates along with longer life expectancy, which in turn promotes a condition of higher proportion of older persons (defined as aged 60 years and over) than any other age group. This phenomenon is called “population aging” (also known as “demographic aging”) by demographers for the shifts in the age distribution (i.e., age structure) of a population toward older ages [1]. Indeed, population aging is now affecting the entire world, which is progressing fastest in developing countries [2, 3]. In the present time, Thailand is an aging society and currently ranked as the second most aged country among the eleven countries in Southeast Asia with the population aged 60 years and over accounted for 17% of the total population, next to Singapore [4, 5]. Besides labor shortages, the major unfavorable consequence of the growing aging population is the rising global burden of chronic non-communicable diseases, particularly chronic neurodegenerative diseases [6, 7]. This leads to the need for long-term care from social services and imposes the greatest burden on health care costs, which has a long-term impact on socio-economic development. Effective management of these chronic diseases may serve as a way of coping with an aged society in the near future.

As the world population ages, neurodegenerative diseases are becoming a major public health concern with increasing incidence and prevalence worldwide with no cure available currently. Neurodegenerative diseases are a group of disorders that occur as a result of chronic and progressive degeneration of neurons in the brain areas specific for each disorder, such as Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's



disease (HD), multiple sclerosis (MS), and amyotrophic lateral sclerosis (ALS). Among a variety of neurodegenerative diseases, the most common and well-known type of age-related neurodegenerative diseases is AD which accounts for approximately two-thirds of all cases. AD is characterized by the progressive loss of neuronal cells in the central nervous system (CNS) leading to an irreversible condition in which neurons gradually deteriorate and lose function which eventually contributes to impaired cognitive abilities, such as learning and memory. Additionally, as the result of abnormal functioning in several areas of the patient's brain in the late stage of disease, AD can be fatal and it is officially ranked as the sixth-leading cause of death in the United States (U.S.) last year [8]. AD is now suffered by nearly 46 million patients around the world, and its prevalence is expected to rise sharply over the next 30 years that the number of AD patients will be more than 130 million globally by 2050 or 1 in 85 persons will be living with the disease [6, 9]. In the absence of an effective treatment, the most commonly used AD drugs include acetylcholinesterase (AChE) inhibitors (e.g., galantamine) to inhibit the AChE enzyme and N-methyl-D-aspartate (NMDA)-type glutamate receptor antagonists (e.g., memantine) to block the activation of the NMDA-type glutamate receptor. Nevertheless, these drugs provide only symptomatic relief but not a cure. The debate over the therapeutic benefits versus the side effects and the financial cost of these drugs has continued for decades [10-14]. The discovery of therapeutic drugs with very high efficacy and fewer side effects is a very active area of neurodegeneration research.

Efforts in searching for new treatment of neurodegeneration have lately been focusing on reducing glutamate-induced neurotoxicity. This mechanism has been implicated in many aging-associated disorders, particularly AD and other neurodegenerative diseases [15]. Disturbance of the glutamatergic system is one of the physiological changes during the normal process of aging in the brain [16-18] that has been hypothesized to play a role in the pathogenesis of numerous age-related neurological conditions including neuronal degeneration [19-22]. Glutamate is the principal and most abundant excitatory neurotransmitter of the mammalian CNS and is involved in a variety of normal brain



functions including cognition, memory, and learning. In the normal condition, glutamate is mainly stored intracellularly inside synaptic vesicles where the concentration may be as high as several millimolar concentrations (up to 100 mM). However, it is inactive until released into the synapse, whereas the concentration of extracellular glutamate is tightly regulated via the uptake through the glutamate transporters to maintain physiological concentrations in the low micromolar range (about 1  $\mu$ M) [23-25]. However, loss of such control under pathological conditions could result in a dramatic rise in extracellular glutamate level that eventually leads to neuronal damage and death. The activities of glutamine synthetase and glutamate transporter were found to be significantly decreased in the brains of individuals with AD, especially in the hippocampus and neocortex [26, 27], suggesting that there was likely an increased extraneuronal accumulation of glutamate since both enzyme and transporter activities are required for glutamate clearance from the synapse. Furthermore, prolonged exposure of high glutamate levels could increase the production of toxic beta amyloid ( $A\beta$ ), a well-known hallmark of AD, by regulating the amyloidogenic processing of the amyloid precursor protein (APP). In turn,  $A\beta$  has been shown to influence glutamate concentration in the synaptic cleft via inhibition of glutamate uptake [28, 29]. There was more evidence of altered glutamate homeostasis in other neurodegenerative diseases. Impaired glutamate reuptake and overactivation of glutamate receptors were implicated in the degeneration of dopaminergic neurons in the substantia nigra, which is a hallmark of PD [15, 20, 30]. Elevated glutamate levels and impaired glutamate clearance were found in the brains of patients suffering from MS, a chronic inflammatory demyelinating disease of the CNS [31-34]. The increase of glutamate in cerebrospinal fluid (CSF) along with the decreases in protein expression and functional activity of glutamate transporters were reported in patients who have ALS, a fatal disease caused by irreversible degeneration of motor neurons [35-39]. One of the main mechanisms underlying the neurotoxic effects of glutamate is an oxidative stress-induced programmed cell death pathway called oxidative glutamate toxicity or oxytosis [40]. In this cell death paradigm, glutamate at pathological levels induces inhibition of cystine uptake



by the cystine/glutamate antiporter (system Xc<sup>-</sup>), which glutamate capable of acting as a competitive inhibitor of cysteine or even reversing the action of system Xc<sup>-</sup> [41, 42]. Such depletion of cysteine leads to the impaired production of the endogenous antioxidant glutathione (GSH) and thereby enhancing accumulation of reactive oxygen species (ROS) as well as oxidative stress. Subsequently, the elevated ROS level disrupts mitochondrial membrane integrity and results in the release of apoptosis-inducing factor (AIF), which eventually triggers neuronal death via a caspase-independent pathway [43, 44]. Therefore, targeting towards glutamate-mediated oxidative toxicity pathway (i.e., suppression of glutamate-induced oxidative stress) may provide a beneficial therapeutic approach for the treatment of AD and other neurodegenerative disorders.

Herbal medicines are gaining significant attention globally in primary health care due to the high cost and adverse effects of synthetic drugs, especially in long-term usage. Synthetic FDA approved drugs may have long-term adverse side effects [45] and are not cost-effective or readily affordable in under developing countries [46, 47]. The use of medicinal plants or plant-derived substances has been proven to be effective in the prevention and/or treatment of several diseases [48-50]. Such illnesses include AD, depression, bacterial infections, type II diabetes, and cancer [51]. Examples of plant-derived FDA approved drugs used for the treatment of AD are rivastigmine and galantamine, which were isolated from *Physostigma venenosum* and *Galanthus caucasicus* [52, 53]. Other promising natural products such as Huperzine A (derived from *Huperzia serrata*), curcumin (derived from *Curcuma longa*), and resveratrol (derived from *Vitis vinifera*) also possess excellent anti-AD activity and they are in Phase II or III clinical trials [54, 55]. Moreover, a number of medicinal plants with antioxidant, anti-inflammatory, and anti-apoptotic effects are currently being researched as an excellent source of neuroprotective agents and/or anti-AD drugs [52, 53, 56-59].

Owing to the hot and humid climate conditions, Thailand is a place known for cultivating a variety of tropical plants and herbs. Therefore, in connection with the



aforementioned pathogenic role of glutamate in triggering neurodegeneration, we set out to investigate the effects of Thai medicinal plant extracts and their underlying mechanisms on the attenuation of glutamate-induced neuronal cell death in the mechanism of oxidative glutamate toxicity using *in vitro* model of neurodegeneration. The underlying neuroprotective mechanisms were studied in the pathways related to anti-apoptosis and antioxidant defenses. Moreover, as neurodegenerative diseases are known to be strongly associated with age, we are interested in examining the effects of Thai medicinal plant extracts on lifespan extension using the nematode *Caenorhabditis elegans* as a model organism. We have also performed the phytochemical analysis to identify putative phytochemical components of the promising extracts that could be responsible for their neuroprotective and/or longevity effects. Furthermore, in order to enhance the utilization of the plant extracts with the best efficacy for therapeutic applications and facilitate the clarification of its active components, an extract that possessed the strongest protective effects against oxidative glutamate toxicity among the studied extracts were isolated into three sub-fractions using liquid-liquid extraction based on pH properties of their phytochemical constituents. These fractions were investigated and compared to each other for their pharmacological potentials including neuroprotective properties. In the present study, the mouse hippocampal neuronal HT-22 cell line was used as a cellular model of neurodegenerative diseases, because in AD the hippocampus is a brain region that has significant loss of neurons and hippocampal atrophy is the best-validated marker of AD [60-62]. In addition, since HT-22 cells are devoid of the NMDA-type ionotropic glutamate receptors [42, 63], the mechanism of oxidative glutamate toxicity could be investigated in isolation from receptor-dependent glutamate toxicity in this cell line. Thai medicinal plants used in this study were selected according to the following criteria: (1) found in Thailand, and (2) never been studied for the effect against glutamate-induced neurotoxicity, and/or (3) traditionally used as an ingredient in Thai traditional formula for longevity or for brain function improvement, and/or (4) available reported for anti-inflammation and antioxidants.



## 1.2 Research questions

1. Do the selected Thai medicinal plant extracts protect neuronal cells against glutamate-induced oxidative toxicity?
2. What are the protective mechanisms of the selected Thai herbal extracts against glutamate-induced oxidative toxicity?
3. Do the selected Thai medicinal plant extracts have anti-aging properties?
4. Which phytochemical constituents or bioactive fractions in the selected Thai herbal extracts are responsible for the neuroprotective and/or anti-aging effects?

## 1.3 Research objectives

1. To examine the protective effects of the selected Thai medicinal plant extracts against glutamate-induced oxidative toxicity using mouse hippocampal neuronal cell model.
2. To investigate whether the neuroprotective effects of Thai herbal extracts are through suppressing glutamate-induced apoptotic cell death via inhibition of mitochondrial release of AIF.
3. To investigate whether the neuroprotective effects of Thai herbal extracts are through lowering glutamate-induced oxidative stress via up-regulation of the Nrf2 signaling pathway.
4. To examine the longevity effects of the selected Thai medicinal plant extracts using the nematode *C. elegans* model.
5. To characterize the phytochemical profiles of the promising extracts by LC-MS analysis.
6. To isolate bioactive fractions from the promising extracts using liquid-liquid extraction based on pH properties of the phytochemical constituents and to compare their antibacterial, antioxidant, anti-AChE, and neuroprotective activities.

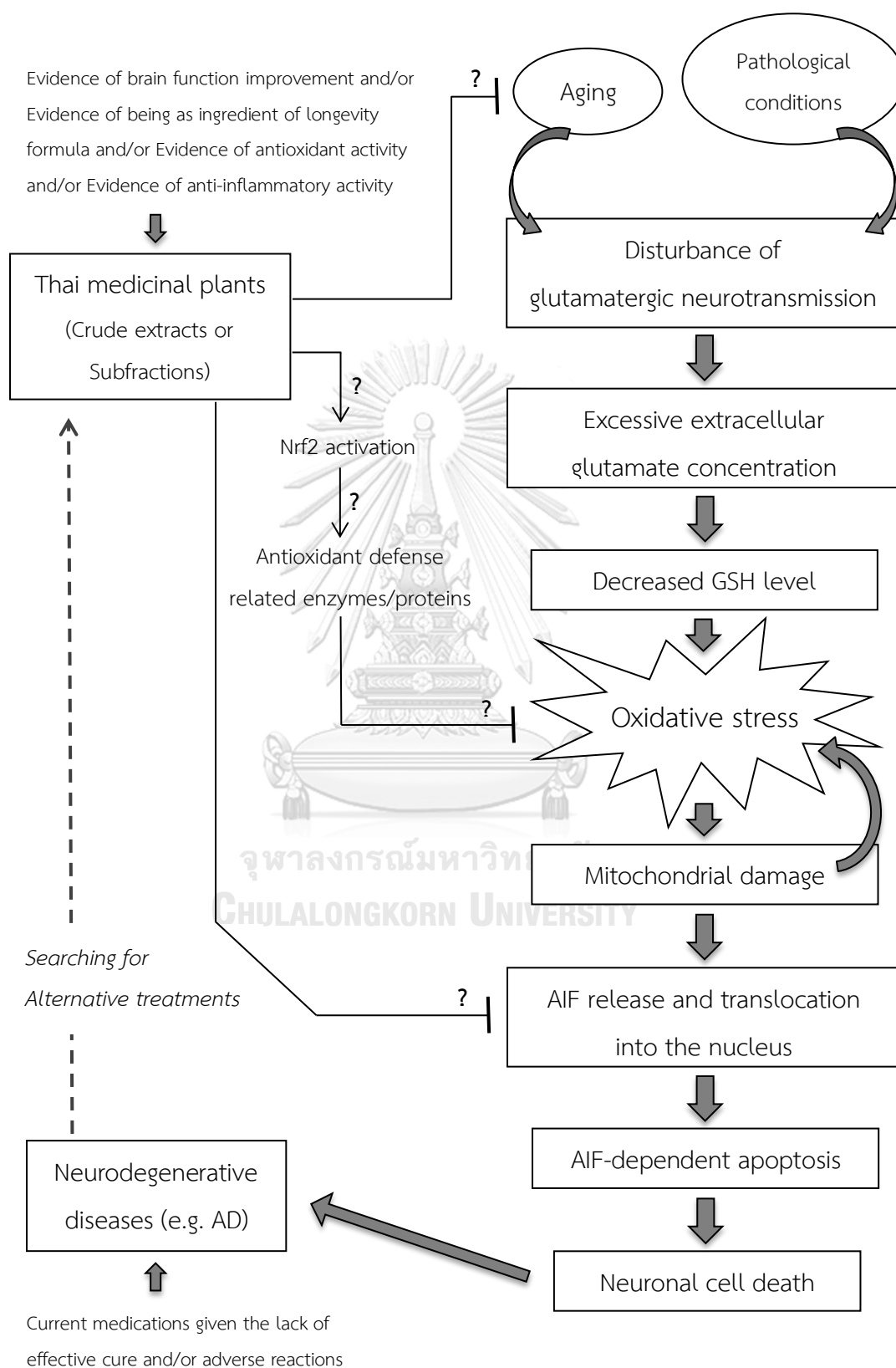


#### 1.4 Research hypotheses

1. One or more Thai medicinal plant extracts can protect against neuronal cell death induced by oxidative glutamate toxicity.
2. The promising extracts can prevent oxidative glutamate toxicity by suppressing apoptotic cell death via inhibition of mitochondrial release of AIF.
3. The promising extracts can prevent oxidative glutamate toxicity by lowering oxidative stress through increased antioxidant defense system via up-regulation of the Nrf2 signaling pathway.
4. The promising extracts can extend the lifespan of *C. elegans*.
5. The promising extracts contain one or more phytochemical components that can be responsible for the neuroprotective and/or anti-aging effects.
6. The promising extracts contain one or more bioactive fractions with multi-therapeutic potentials that have higher protective activity against oxidative glutamate toxicity than that of crude plant extracts, and possess either anti-AChE or antibacterial properties.

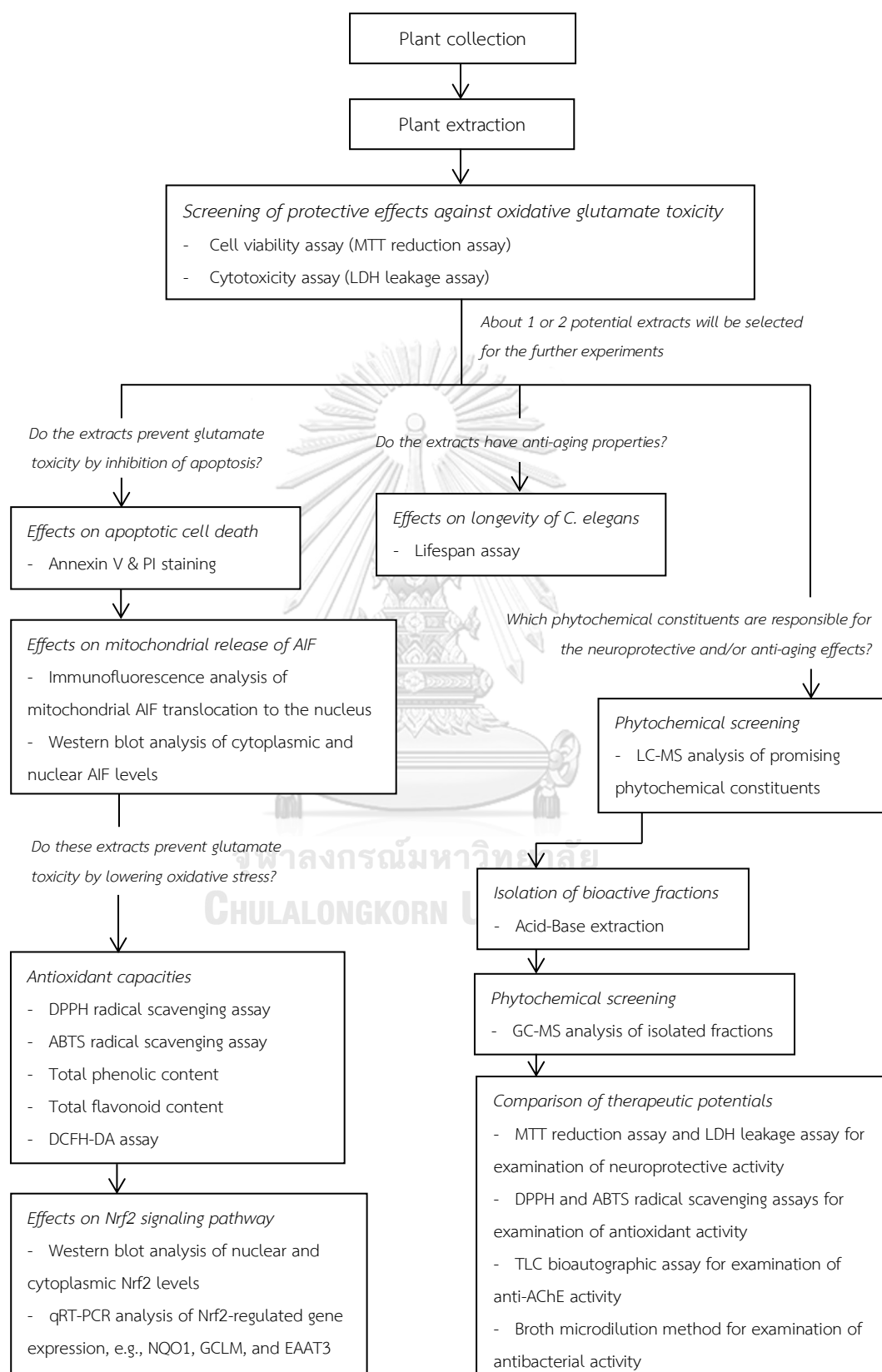


## 1.5 Conceptual framework





## 1.6 Experimental design





## CHAPTER II

### LITERATURE REVIEW

#### 2.1 Population aging

Given the tremendous achievement of public health care programs and the significant progress in socio-economic development over the past century, the average life expectancy at birth of the global population has increased dramatically with falling mortality rates. As a result, the proportion of older people (aged 60 years and over) is growing faster than any other age group. This phenomenon is called “population aging” (also known as “demographic aging”) by demographers for the shifts in the age distribution (i.e., age structure) of a population toward older ages [1]. Indeed, this population ageing is now taking place in nearly all the countries of the world but is more advanced in less developed regions with about two-thirds of the world’s older persons live in developing countries [2, 3]. The rate of population ageing is likely to accelerate quite rapidly over the next few decades. Globally, the proportion of population aged 60 years and older is projected to grow faster than younger aged group from an estimated 13% in 2017 to nearly 25% in 2050 or the number of people aged 60 and over is expected to increase more than double from an estimated 962 million to almost 2.1 billion over the same period [3]. Thailand’s population is also rapidly ageing as the older persons currently constitute 17% of the total population, making Thailand ranked as the second most aged country among the eleven countries in Southeast Asia next to Singapore, and the 63rd worldwide [3-5]. The proportion of older persons in Thailand is projected to increase to nearly 30% or exceed 20 million by 2050 [4].

As the age structure shifts more towards higher age group, there would be fewer persons of working age to support an increasing number of older persons. Such a demographic change can have unfavorable impacts on social and economic processes



[64]. The major consequence is the slowdown in the growth rates of labor productivity and gross domestic product (GDP) due to shrinkage of the working-age population. This ageing phenomenon may also negatively influence the pension system. Additionally, the risks of disability and non-communicable diseases (e.g. cancer, diabetes, obesity, cardiovascular diseases, stroke, and neurodegenerative diseases) that more commonly affect adults and older people also increase as population ages [65]. This leads to the need for long-term care from social services and imposes the greatest burden on health care costs, which has a long-term impact on socio-economic development. Therefore, in order to cope with this difficult situation, there is an urgent need for the research and development of effective therapies against major aging-related diseases, particularly incurable neurodegenerative diseases which are becoming more common in an ageing population [6, 7, 66]

## 2.2 Neurodegenerative diseases

Neurodegenerative disease is a generic term used for a wide range of incurable and debilitating condition characterized clinically by loss of neurological function (dementia, loss of movement control, paralysis), and pathologically by progressive degeneration or death of nerve cells, particularly those in the central nervous system (CNS) [67]. They include Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD), amyotrophic lateral sclerosis (ALS), Creutzfeldt-Jakob disease (CJD), motor neuron disease (MND), prion disease, and multiple sclerosis (MS). Of these, AD is the most common form and responsible for the greatest burden of this disease group, which accounts for approximately two-thirds of all cases [8]. The second most common neurodegenerative disease is PD [68]



## 2.3 Alzheimer's disease

Alzheimer's disease (AD) is the most common neurodegenerative disease named by the German physician Dr. Alois Alzheimer in 1906 [69], which results from progressive and irreversible deterioration of neuronal structures and brain functions. It is also known as the most common cause of dementia, a clinical condition in which an individual's cognitive functions deteriorate accompanied by behavioral and psychological abnormalities [70]. AD normally develops differently for the individual who presents both shared and unique symptoms that usually reflect the degree of damage to neurons in different parts of the brain, from mild to very severe [8, 71]. In the early stages, the most common defect is remembering recent events or short-term memory impairment. But, AD is more than just memory loss. As the disease progresses, AD patients gradually lose their ability to think and reason clearly, make judgments, solve problems, communicate, and take care of themselves. Symptoms also include confusion, irritability and aggression, mood swings, changes in personality and behavior, problems with attention and spatial orientation, trouble with language, and long-term memory loss, all of which can affect a person's daily life. AD can even lead to the death of the afflicted person in the final stages by causing malnutrition, brain death, and multiple organ failure due to the number of nerve cells that have died. AD can occur in anyone at any age, however, the vast majority of AD patients are the elderly who aged 65 and over (about 95% of all cases) and it is less prevalent in younger people [72]. At present, AD afflicts nearly 46 million worldwide, and its prevalence is rising each year dramatically due to the longer life expectancy worldwide. By 2050, the number of individuals with AD is expected to quadruple to more than 106 million globally, and it is estimated that 1 in 85 persons will be living with the disease [6, 9]. Likewise, the number of AD patients in Thailand was expected to rise over a million by 2030 from around 600,000 people, announced by the Ministry of Public Health in 2016.



### 2.3.1 Diagnosis of Alzheimer's disease

The gold standard for a “definite” diagnosis of AD is an autopsy-based neuropathological evaluation by examining the presence of senile (neuritic) amyloid plaques and neurofibrillary tangles (NFTs) in the post-mortem brain, which constitute hallmarks of AD (Figure 1) [73, 74]. However, these lesions are often found in the later stage of AD disease while the diagnosis should be made at the early stage to provide patients with the best opportunities for treatment. Currently, in the regular clinical practice, the diagnosis of AD is instead performed largely based on clinical data with the sensitivity of 70-90% and specificity of 50-70% relative to standard pathological diagnosis, and commonly defined as “probable” AD dementia (dementia as a consequence of AD) [73, 75]. A clinical diagnosis for AD is not a single test, but is usually made through a series of assessment tests that considers all possible causes and rules out other medical issues which may cause symptoms similar to that of AD, such as brain tumor, stroke, depression, vitamin deficiency, and thyroid disease [8]. The methods include obtaining the patient's medical and family history, mental and physical examinations, brain scans (e.g. magnetic resonance imaging (MRI), computerized tomography (CT), positron emission tomography (PET)), neurological evaluations (e.g. reflexes, sensation, eye movement, speech), electroencephalography (EEG) testing, cognitive and other neuropsychological assessments (e.g. the Mini-Cog test, the Mini-Mental State Examination (MMSE), the Alzheimer's Disease Assessment Scale-Cognitive subscale (ADAS-Cog), the Geriatric Depression Scale (GDS)), laboratory testing (e.g. thyroid-stimulating hormone (TSH) level, vitamin B12 level, folate level), as well as genetic testing (e.g. the  $\epsilon 4$  allele of apolipoprotein E (APOE) gene, the mutations of amyloid precursor protein (APP), presenilin 1 (PSEN1), and presenilin 2 (PSEN2) genes [76]. Until now, several different sets of diagnostic criteria for AD have been released and undergone multiple revisions [77]. Three recent guidelines for diagnosis of AD in use are the second edition of the IWG criteria (IWG-2) published by an International Working Group in 2014 [78], the fifth edition of the Diagnostic Statistical Manual of Mental Disorders criteria (DSM-5) published by the American Psychiatric Association in 2013 [79,



80], and the NIA-AA criteria published by the National Institute on Aging and the Alzheimer's Association in 2011 [81, 82].

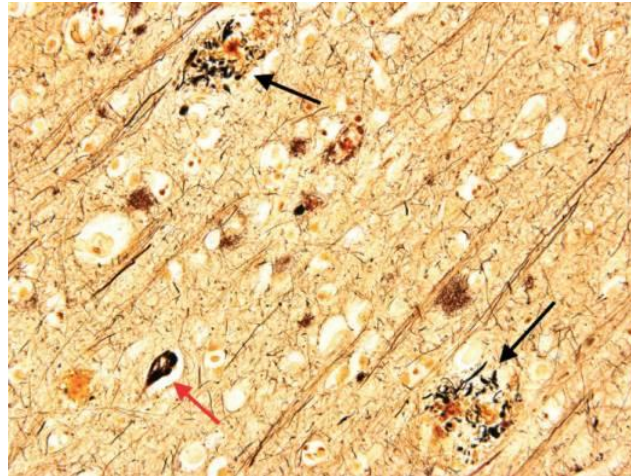


Figure 1: Amyloid plaques and neurofibrillary tangles.

Amyloid plaques (black arrows) and neurofibrillary tangles (red arrow) in the AD-affected brain, revealed by the Bielschowsky silver stain. (Image from [83])

### 2.3.2 Causes of Alzheimer's disease

Over the past several decades of research, AD is now considered as a complex and multifactorial disease as the actual cause of AD remains inconclusive, even though its histopathological features in the brain are well characterized. Typically, AD can be classified as rare early-onset and more common late-onset forms in case of the disease occurring before and after age 65 [84]. Several modifiable (e.g. diet) and non-modifiable (e.g. genetics) factors are associated with the development and progression of both AD forms and this may differ from patient to patient [85]. Until now, the etiology of AD has been explained by many theories involving genetics, brain chemicals, misfolding and aggregation of key proteins, environmental or external stimuli, as well as aging, lifestyle and health problems [86]. Most of the well-known etiological factors along with their roles in the in the pathogenesis of AD are summarized below.



### 2.3.2.1 Etiological factors in the pathogenesis of Alzheimer's disease

#### 2.3.2.1.1 Genetics

AD can be caused by a genetic defect transmitted from generation to generation, called familial AD. The rare early-onset familial AD which accounting for less than 2% of total cases is developed as a result of mutations in specific genes inherited in an autosomal recessive pattern, while the rest do not have a clear pattern of inheritance [76, 87, 88]. To date, three causative genes responsible for the early-onset familial AD has been identified including genes on chromosomes 21, 14, and 1, coding for the amyloid precursor protein (*APP*) and the presenilin 1 and 2 proteins (*PSEN1* and *PSEN2*), respectively [87]. These mutations affect the processing of APP, leading to increased production of amyloid beta ( $A\beta$ ) or altered production of different  $A\beta$  peptide species in favor of toxic forms which are more aggregation-prone [76]. Interestingly, there is a strong connection between AD and Down syndrome, a genetic disorder caused by the presence of an additional copy of chromosome 21 (also known as Trisomy 21). People with Down syndrome are at higher risk of developing AD that could be resulted from overexpression of APP gene carried on chromosome 21 which is in triplicate [89]. In contrast to early-onset form, the genetic architecture of late-onset AD is more complex as several variants in multiple genes have been implicated in this form by genome-wide association studies (GWAS) [90, 91]. However, thus far the top identified genetic risk factor for both early- and late-onset AD is Apolipoprotein E (*APOE*) gene on chromosome 19 [92], which each APOE isoforms differentially regulate  $A\beta$  aggregation and clearance in the brain [93, 94]. Individuals carrying the *APOE*  $\epsilon 4$  allele that encodes the isoform E4 have a gene-dose effect on increasing risk of developing AD (estimated to be 3 to 15 fold) and lowering the onset age [76, 95, 96].



#### 2.3.2.1.2 Age, Gender, Health, Lifestyle, and Environmental factors

Apart from APOE, the age and gender are other two more well-documented non-modifiable risk factors for the late-onset AD, where its incidence was found significantly greater in women and advancing age [97]. Other known potential risk factors for developing AD include health problems (e.g. traumatic brain injury, cardiovascular disease, obesity, diabetes, depression, hypertension, and stroke), lifestyle (e.g. lack of physical activity, smoking or exposure to secondhand smoke), poor diets (e.g. high cholesterol, high sugar, and high alcohol intake, low fruits and vegetables consumption), low educational level, and environmental contaminants (e.g. toxic metals such as lead, arsenic and aluminum, pesticides/insecticides, and air pollutants) [8, 98-100].

#### 2.3.2.1.3 Neurotransmitters

Neurotransmitters are endogenous chemical substances used by the nervous system to act as the messengers for communication between adjacent neurons via transmission of these chemical signals from one neuron to the next across specialized gap junction called the synaptic cleft. The neurotransmitters are essential for controlling a wide variety of both physical and psychological functions including heart rate, blood pressure, body movement, sleep, appetite, mood and emotional response, as well as learning and memory formation. Hence, disturbance of neurotransmitter systems can have many deleterious effects that are involved in the etiology of AD. The earliest theory of AD pathogenesis lines in the first neurotransmitter discovered, acetylcholine (ACh) which plays an important role in processing memory and learning [101]. The researchers observed a significant reduction of cholinergic neurons and choline acetyltransferase (ChAT), an enzyme responsible for the synthesis of ACh, as well as reduced uptake of choline, a precursor for ACh synthesis, in the brains of AD patients [102]. It was thought that reduction of cholinergic neurotransmission in both concentration and function of ACh led to a decline in cognitive abilities, behavioral and psychological changes seen in AD, and



these deficits may be alleviated through inhibition of acetylcholinesterase (AChE), an enzyme which catalyzes the hydrolysis of ACh, resulting in increased ACh levels in the synaptic cleft [103]. The neurotransmitter serotonin that plays a pivotal role in mood and cognition was recently pointed out as another key player in driving the progression of AD, rather than being an effect of disease [104]. Compared to healthy controls, previous studies showed that AD patients have degeneration of the serotonergic system, as revealed by reduction of serotonin and its metabolites levels, decreased expression of serotonin receptors, as well as substantial loss of serotonergic neurons in AD patients with severe cognitive decline [105]. A greater reduction of serotonin transporters was also observed in the brains of people with a mild cognitive impairment which is a very early sign of AD [104]. These findings suggested that lower serotonin levels in the brain may underlie impaired cognition at the pre-clinical stage of AD. Furthermore, our present research focused on glutamate which is the most abundant neurotransmitter found in the human brain and plays a key role in long-term potentiation [106, 107]. An increasing body of evidence also implicates the role of glutamate in AD, which is detailed in the following sections 2.4 and 2.5.

#### 2.3.2.1.4 Amyloid beta

So far, most of the researchers have accepted the amyloid hypothesis as the leading theory behind AD, in which the amyloid beta ( $A\beta$ ) plays a central role in the development of the disease. The neuropathological processes related to AD are believed to be triggered by the aberrant accumulation and deposition of these  $A\beta$  peptides in the brain [108]. The  $A\beta$  peptide was initially identified and biochemically characterized in 1984 [109] as a peptide that aggregated and was deposited outside neurons in the brain tissue of AD patients, leading to the formation of senile (neuritic) plaques (also called amyloid plaques) in the AD brain. The presence of these plaques is the major pathological hallmark of AD. The  $A\beta$  peptide is a 4.2 kDa short peptide of mainly 40–42 amino acids, generated from the intracellular cleavage of the amyloid precursor protein (APP) by the sequential



action of two proteolytic enzymes, beta- ( $\beta$ -) secretase and gamma- ( $\gamma$ -) secretase. A schematic of the normal proteolytic processing of APP is shown in Figure 2. These soluble monomeric A $\beta$  fragments can further aggregate into various sized oligomers and insoluble fibrils, which subsequently form insoluble amyloid plaques.

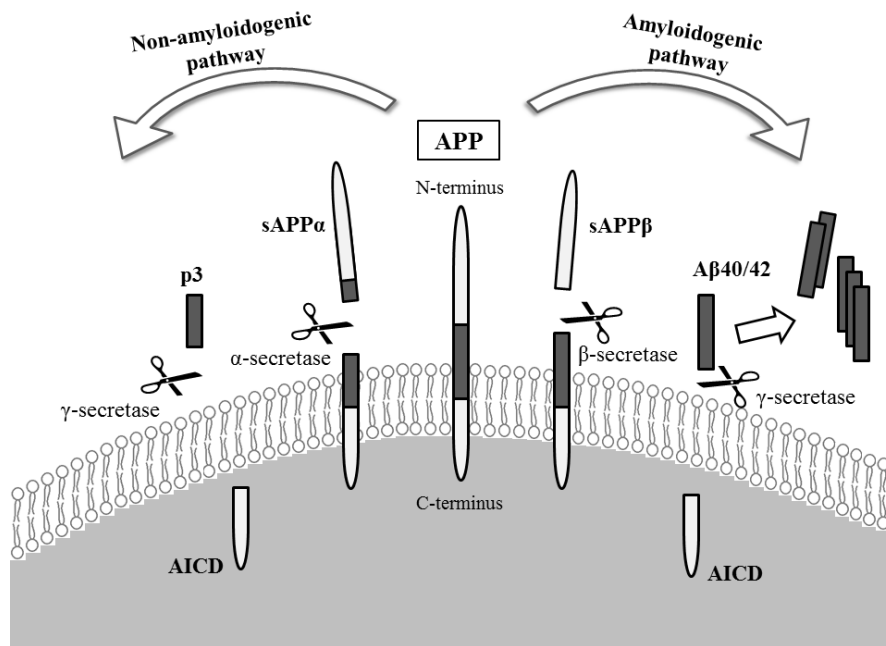


Figure 2: Scheme illustrating the proteolytic processing of APP in two distinct pathways.

APP is a ubiquitously expressed integral membrane protein. In the non-amyloidogenic pathway (left), APP is cleaved within the A $\beta$  domain by the  $\alpha$ -secretase enzyme. However, in the amyloidogenic pathway, APP is first cleaved by  $\beta$ -secretase (BACE1), instead of  $\alpha$ -secretase, at the N-terminus of the A $\beta$  domain, and this is followed by  $\gamma$ -secretase cleavage at the C-terminus. This sequence of events generates the A $\beta$  amyloidogenic peptides, which can aggregate into oligomers and form extracellular neurotoxic plaques in the brain. Both pathways release identical APP intracellular C-terminal domain (AICD) (Image from [108]).

Indeed, the A $\beta$  peptides are normally produced by astrocytes and neurons. They are usually presented in a soluble form and serve several important physiological



functions that are beneficial for the brain [110, 111]. In contrast, when these peptides form aggregated structures, they become toxic to brain cells by inducing changes in several biochemical molecules, thereby causing many deleterious events related to AD pathology including membrane disruption, proteasome impairment, Tau hyperphosphorylation, mitochondrial dysfunction, inflammation, oxidative stress, synaptic failure, and ultimately cell death [112-114]. However, under physiological conditions, our body can control the A $\beta$  level through various mechanisms. In the healthy brain, the concentration of the A $\beta$  peptide is regulated by its production from APP and influx into the brain across the blood-brain barrier (BBB), mainly via the receptor for advanced glycation end products (RAGE), and by clearance from the brain via the low-density lipoprotein receptor-related protein-1 (LRP1) and enzymatic degradation within brain [115-117]. Thus, impairment of these regulatory mechanisms could lead to the accumulation and deposition of excessive amounts of A $\beta$  peptide and their aggregates in the brain, which subsequently develops a series of pathological alterations of AD [108].

#### 2.3.2.1.5 Tau protein

Along with the A $\beta$  peptide, the microtubule-associated protein Tau, which is primarily located in neuronal axons, is thought to be another key driver of AD. Under normal physiological conditions, Tau performs critical functions in promoting the assembly and stability of microtubules that are essential for the maintenance of neuronal cytoskeleton and axonal transport [118]. However, in pathological conditions such as AD, microtubule-bound Tau undergoes excessive or abnormal phosphorylation and dissociates from microtubules resulting in increased soluble free Tau. These unbound phosphorylated tau proteins tend to self-aggregate into insoluble paired helical filaments (PHFs) forming intraneuronal neurofibrillary tangles (NFTs) [119]. This insoluble structure and aggregated forms of Tau proteins cause neuronal damage by interfering with microtubule-based neuronal transport mechanisms, altered distribution and function of organelles, particularly mitochondria that are essential for cell survival, ultimately leading to neuronal death [120].



#### 2.3.2.1.6 Oxidative stress

Oxidative stress is classically described as an imbalance of redox homeostasis, resulting from the overproduction of free radicals relative to the innate ability of cells to scavenge them. This detrimental event causes damage to cellular components and alterations in cellular function that ultimately contribute to cell death [121, 122]. Reactive oxygen species (ROS) are the most common type of free radicals produced in the human body and play an important role in cellular injury in various tissues, particularly the brain, which is highly sensitive to oxidative damage due to its large dependence on oxygen consumption [123, 124] and its high abundance of lipids susceptible to peroxidation [125]. Oxidative stress has long been associated with aging and age-related neurodegenerative diseases including AD, in which the action of ROS is implicated in the mechanism of neuronal loss [126, 127]. Under oxidative stress conditions, elevated ROS induces mitochondrial dysfunction including reduced energy production, calcium dyshomeostasis, loss of mitochondrial membrane potential, perturbation in mitochondrial dynamics, damage or mutations in mitochondrial DNA, as well as inhibition of electron transport chain (ETC) and more ROS release, creating a vicious circle of ROS-induced ROS production [128]. Along with oxidative stress, these alterations in mitochondrial function are recognized as early pathological events and as underlying mechanisms of neuronal cell death in AD [129, 130]. For example, caspase-dependent apoptosis can be triggered by cytochrome c that released from mitochondria to the cytosol as a consequence of calcium-induced mitochondrial membrane potential collapse [131]. Moreover, oxidative stress can aggravate the damage in AD brain by increased production of A $\beta$  possibly through promoting the expression and activity of beta- and gamma-secretases, enzymes critical for A $\beta$  generation [132, 133]. Oxidative stress may also promote the phosphorylation and aggregation of tau protein possibly via glycogen synthase kinase-3 $\beta$  (GSK-3 $\beta$ ) activation [134, 135]. On the other way round, the A $\beta$  itself has been shown to be another critical source of ROS. The metal ions (primarily iron (Fe) and copper (Cu)) that



generally bound to A $\beta$  species are potent catalysts of ROS generation via Fenton chemistry [136, 137].

Due to its high reactivity, the ROS can directly cause oxidative damage to cellular structures and biomolecules that subsequently lead to cell death [27, 138, 139]. There was evidence of enhanced ROS-induced oxidative modifications of proteins (e.g. protein carbonyl), lipids (e.g. 4-hydroxynonenal), and nucleic acids (e.g. 8-hydroxydeoxyguanosine) in AD brain [140-142]. Proteomic studies have identified several oxidatively modified proteins in AD [143, 144]. For example, oxidized ubiquitin carboxy-terminal hydrolase L-1 (UCH L-1) leads to proteasomal dysfunction and the consequent accumulation of damaged, misfolded, and aggregated proteins. Oxidatively modified creatine kinase BB (CK) and glutamine synthetase (GS) severely affect ATP production and the influx of calcium ions into neurons, resulting in the loss of function of ion pumps, the dysregulation of intracellular calcium homeostasis, alterations in LTP, and mitochondrial dysfunction with the release of proapoptotic factors. Interestingly, ROS also can activate signal transduction of redox-sensitive transcription factors, such as nuclear factor- $\kappa$ B (NF- $\kappa$ B) that regulates the expression of several genes involved in the inflammatory response [145]. To date, many compounds, specifically naturally derived substances, which possess powerful antioxidant and/or free radical scavenging activities have been suggested to be effective in treating such oxidative stress-related diseases including AD [146-148].

#### **2.3.2.1.7 Inflammation**

Inflammation is a part of the complex biological response of the body to any harmful stimuli such as pathogens, toxins, and damaged tissues. However, uncontrolled or abnormal activation of inflammatory mediators in the nervous system (often termed neuroinflammation) can have detrimental effects leading to neuronal death and neurodegeneration. Considerable evidence has implicated neuroinflammation early in the pathogenesis of AD, although it remains unclear whether inflammation in AD is a primary



cause or a secondary phenomenon [149-151]. Inflammatory components related to neuroinflammation in AD include the major glial cells of the CNS, astrocytes and microglia. The reactive (activated) forms of these cells were found in close association with amyloid plaques [152], the classical and alternative complement systems which appears to be activated in AD and their components (e.g. C1q and C3) were found colocalized with amyloid deposits [153, 154], as well as the pro- and anti-inflammatory cytokines such as interleukin-1 $\beta$  (IL-1 $\beta$ ), IL-4, IL-6, tumor necrosis factor-alpha (TNF- $\alpha$ ), and transforming growth factor-beta (TGF- $\beta$ ) which their expression levels were found altered in AD brains [155]. Likewise, a series of large-scale genomic and transcriptomic studies strongly support the role of inflammatory processes in AD pathogenesis, particularly functions of microglia, the resident immune cells in the brain [156-158]. Hence, anti-inflammatory approaches may provide effective options for the prevention and therapy of AD [159].

#### 2.3.2.1.8 Microbial infection

There is a growing body of evidence supporting the microbe hypothesis that AD could be arising from microbial infection. Previous epidemiological studies showed that infection had been associated with the development and progression of dementia and AD [160-162]. Recent evidence using next-generation sequencing (NGS) analysis that observed an increase in bacterial populations in post-mortem brains from patients with AD, also supports the involvement of bacterial infection in AD [163]. Until now, a number of specific viruses, bacteria and fungi have been detected and implicated in AD [164-166]. For example, the herpes simplex virus type 1 (HSV-1), the most common type of HSV infection known to cause cold sores on the mouth [167], *Chlamydomydia pneumoniae*, a gram-negative bacteria known to be a major cause of pneumonia [168, 169], *Helicobacter pylori*, a spiral gram-negative bacteria known to cause chronic gastritis and peptic ulcers [170, 171], and Spirochetes, a group of gram-negative bacteria having an unusual helical and flexible shape known to cause syphilis [172]. However, it is still unclear how these



pathogens cause AD. One possible common mechanism is that the pathogens trigger chronic inflammation in the brain that may subsequently promote the initiation and progression of the disease [173-175]. On the other hand, the presence of pathogens in the brain may lead to excessive accumulation and deposition of A $\beta$  peptides, the major pathological hallmark of AD, which these peptides may be recruited to the site of infection due to their functions in innate immunity as antimicrobials [176, 177] and antivirals [178, 179]. Although much remains to be determined about the mechanisms linking infections to AD, this emerging hypothesis might provide an alternative strategy for disease prevention and treatment by controlling AD-associated infections [174, 180-182].

### 2.3.3 Treatments of Alzheimer's disease

AD causes the shrinkage of many parts of the brain where the neurons and their connections are progressively deteriorated. As such, there is no cure for AD because the death of brain cells cannot be completely halted and reversed by currently available medications. Nevertheless, those drugs can provide symptomatic relief, although to some but not all patients, in managing behavioral, mental and cognitive problems, and thereby help improve the quality of life for AD patients and their caregivers as well. Currently, there are only five medications approved by the U.S. Food and Drug Administration (FDA) for treating the cognitive symptoms and/or problematic behaviors in AD [183] (Figure 3), which three of them are in a class called cholinesterase inhibitor, i.e., donepezil (Aricept®), galantamine (Reminyl®), and rivastigmine (Exelon®). These drugs prevent the breakdown of acetylcholine, a chemical messenger in the brain, which is important for learning and memory, by inhibiting the activity of enzyme acetylcholinesterase (AChE). Another medication is memantine (Namenda®), an *N*-methyl-D-aspartate (NMDA) receptor antagonist, which works by regulating the activity of glutamate, the major brain's neurotransmitter involved in cognitive functions, by blocking NMDA-type glutamate receptors. The latest medication approved in 2014 is a fixed-dose combination drug of



donepezil and memantine (Namzaric®). In general, donepezil is approved to treat all stages of AD, while galantamine and rivastigmine are recommended to treat patients with mild to moderate stages of AD, and memantine is approved to treat patients with moderate to severe stages of AD [183].

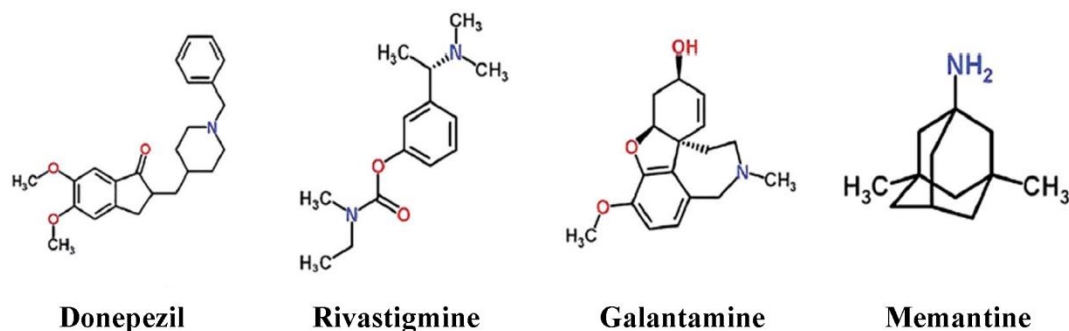


Figure 3: Chemical structures of drugs approved by the FDA for treatment in AD.

(Image from [184])

With a goal to improve treatment of AD, it is very challenging for researchers to develop new “disease-modifying” drugs that help AD patients slow down and stop the progression of neurodegeneration, or even reverse the process of deterioration to get the patient’s brain back to the point of healthy. Not only therapy, development of preventive strategies for AD is also another important point to take into account. It is better if the disease can be detected and prevented before the symptoms occur or become severe. Regarding an unsuccessful cure with currently prescribed drugs along with a long history of traditional use of herbal medicines, many different plants (or herbs) are regaining significant attention globally as alternative medicine and also as a potential source of natural substances for therapeutic and preventive applications in several different diseases [51]. In line with drug development for AD, numerous medicinal plants and plant-derived substances have been studied and proved to have benefits against the disease [185]. In fact, two FDA-approved drugs, galantamine and rivastigmine, are derived from the plants,



*Galanthus caucasicus* and *Physostigma venenosum*, respectively [52, 186]. Other promising natural products such as Huperzine A (derived from *Huperzia serrata*), curcumin (derived from *Curcuma longa*), and resveratrol (derived from *Vitis vinifera*) also possess excellent anti-AD activity and they are in Phase II or III clinical trials [54, 55]. Moreover, a number of medicinal plants and pure compounds have been extensively researched for their neuroprotective and anti-AD potentials [52, 53, 56-59, 184, 185].

In the past few decades of drug development for AD, most therapeutic strategies have been focused majorly on amyloid beta ( $A\beta$ ) formation cascade that has been widely accepted as a key etiological pathway in the pathogenesis of the disease [182, 187, 188]. Most of the efforts have been made in the modulation of  $A\beta$  formation, aggregation, and clearance. However, there is still much effort in the development of new cholinesterase inhibitors and NMDA receptor antagonists that can provide better efficacy and safety than previous ones [182, 186]. Other disease-modifying approaches that are in active investigations include inhibition of Tau hyperphosphorylation, lowering inflammation in the brain, and boosting the body's antioxidant defense for neuroprotection [182, 184, 187, 189-191]. However, in reality, the development of effective medications for AD is challenging with the disappointing failures of many previous agents in clinical trials [187, 192, 193], highlighting the urgent need to fully understand pathogenic mechanisms of AD to enable more rational drug design. Table 1 summarizes the strategies currently being used in drug development for AD therapy and prevention according to the drug target and the mechanism of action.



Table 1: An overview of therapeutic and preventive strategies targeting five major etiological factors in current AD drug development.

<i>Strategy</i>	<i>Treatment (Agents in clinical trials)</i>
<b>A<math>\beta</math> cascade</b>	
Inhibiting A $\beta$ formation	<ul style="list-style-type: none"> <li>• Beta-secretase inhibitor (e.g. Lanabecestat or AZD3293 [194, 195], Verubecestat or MK-8931 [196], Elenbecestat or E2609 [187], CNP520 [51], LY3202626 [197])</li> <li>• Gamma-secretase inhibitor (e.g. NGP 555 [198], Begacestat or GSI-953 [199], Pinitol or NIC5-15 [200, 201])</li> <li>• Selective inhibitor of APP translation (e.g. Posiphen [202], Phenserine [203])</li> </ul>
Decreasing A $\beta$ aggregation	<ul style="list-style-type: none"> <li>• Amyloid aggregation inhibitor (e.g. KHK6640 [187], Scyllo-inositol or ELND005 [204], APAN or PPI-1019 [205], Caprospinol or SP-233 [205], EGCG [206])</li> <li>• Metal (Zn<sup>2+</sup>/Cu<sup>2+</sup>) chelator (e.g. PBT-2 [207], Clioquinol [207, 208])</li> </ul>
Enhancing A $\beta$ clearance	<ul style="list-style-type: none"> <li>• Monoclonal antibody to A<math>\beta</math> (e.g. Solanezumab [209], Aducanumab [210], Crenezumab [211, 212], Gantenerumab [213])</li> <li>• Polyclonal antibody to A<math>\beta</math> (e.g. Octagam 10% IVIG [214])</li> <li>• A<math>\beta</math> vaccine (e.g. ACC-001 [215], CAD106 [216], UB-311 [217], Affitope AD02 [218])</li> <li>• RAGE inhibitor (e.g. Azeliragon [219, 220])</li> <li>• Microglial P2Y6 receptor agonist (e.g. GC021109 [191])</li> </ul>
<b>Neurotransmitters</b>	
Inhibiting AChE activity	<ul style="list-style-type: none"> <li>• AChE inhibitor (e.g. Huperzine A [54], Memogain or GLN-1062 [221], Phenserine [203])</li> </ul>
Enhancing cholinergic function	<ul style="list-style-type: none"> <li>• <math>\alpha 7</math>-nAChR agonist (e.g. Nicotine [187])</li> <li>• Muscarinic M1 receptor agonist (e.g. NGX267 [222], TAK-071 [187], HTL0009936 [187])</li> </ul>



Counterbalancing the action of glutamate	<ul style="list-style-type: none"> <li>• GABA receptor agonist (e.g. Radequinil or AC-3933 [223], PXT864 [224])</li> </ul>
Blocking glutamate receptors	<ul style="list-style-type: none"> <li>• NMDA receptor antagonist (e.g. Neramexane [225])</li> <li>• AMPA receptor modulator (e.g. Tulrampator or S47445 [226])</li> </ul>
Inhibiting glutamate release	<ul style="list-style-type: none"> <li>• Glutamate release inhibitor (e.g. Riluzole [227])</li> </ul>
<b>Tau protein</b>	
Inhibiting Tau hyper-phosphorylation	<ul style="list-style-type: none"> <li>• GSK3<math>\beta</math> inhibitor (e.g. Valproate [228])</li> <li>• Tyrosine kinase inhibitor (e.g. Nilotinib [229])</li> </ul>
Inhibiting Tau fibrillization	<ul style="list-style-type: none"> <li>• Tau aggregation inhibitor (e.g. Methylene Blue or TRx0237 [190, 230])</li> </ul>
Enhancing Tau clearance	<ul style="list-style-type: none"> <li>• Monoclonal antibody to Tau (e.g. ABBV-8E12 [231], RO7105705 [212], LY3303560 [232])</li> <li>• Tau vaccine (e.g. AADvac1 [233], ACI-35 [234])</li> </ul>
Stabilizing microtubule	<ul style="list-style-type: none"> <li>• Tubulin depolymerization inhibitor (e.g. TPI 287 [190])</li> </ul>
<b>Inflammation</b>	
Suppressing inflammatory processes	<ul style="list-style-type: none"> <li>• NSAID (e.g. ALZT-OP1 [235])</li> <li>• PPAR-gamma agonist (e.g. Pioglitazone [236], Telmisartan [237])</li> <li>• Soluble (Decoy) TNF-alpha receptor (e.g. Etanercept [238])</li> <li>• Inhibitor of TNF-alpha production (e.g. Thalidomide [239])</li> <li>• RAGE inhibitor (e.g. Azeliragon [219, 220])</li> <li>• Natural agents with anti-inflammatory properties (e.g. Curcumin [240], EGCG [206], Omega-3 unsaturated fatty acids plus alpha lipoic acid [241, 242])</li> <li>• Antibiotic (e.g. Minocycline [243, 244])</li> </ul>
<b>Oxidative stress</b>	
boosting the body's antioxidant defense	<ul style="list-style-type: none"> <li>• Antioxidant (e.g. Curcumin [240], Vitamin E [245], <i>Ginkgo biloba</i> extract or EGb761 [246], Resveratrol [247], Echinacoside or STA-1 [187, 248], EGCG [206])</li> </ul>



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Abbreviations: A $\beta$ , Amyloid beta; AChE, Acetylcholinesterase; APP, Amyloid precursor protein;  $\alpha$ 7-nAChR, Alpha7 nicotinic acetylcholine receptor; AMPA, Alpha-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; EGCG, Epigallocatechin-3-gallate; GABA, Gamma-aminobutyric acid; GSK3 $\beta$ , Glycogen synthase kinase 3beta; NMDA, *N*-methyl-d-aspartate; NSAID, Non-steroidal anti-inflammatory drug; PPAR, Peroxisome proliferator-activated receptor; RAGE, Receptor for advanced glycation end product; TNF, Tumor necrosis factor.

## 2.4 Glutamatergic system

Glutamate is an anion form of glutamic acid, a non-essential amino acid. Not only can it be synthesized in our body, but is also found abundantly in both plant and animal sources. Generally, glutamate has probably been well known as "monosodium glutamate" or "MSG" which is used as a flavor or taste enhancer in food (called the umami taste) [249]. In human, glutamate plays an essential role in many physiological functions in addition to protein synthesis. It is an important molecule involved in cellular metabolism as the main product of transamination reaction in the breakdown of amino acids for energy and as a precursor of  $\alpha$ -ketoglutarate, a key intermediate in the tricarboxylic acid (TCA) cycle, or the Krebs cycle when it undergoes oxidative deamination [250]. It also plays a critical role in nitrogen balance as a compound that helps in the elimination of toxic ammonia from the body [251]. Glutamate can have a role in antioxidant defense system by forming a major cellular antioxidant GSH together with cysteine and glycine [252]. Moreover, glutamate has been recognized as the major excitatory neurotransmitter in the mammalian CNS, particularly the cerebral cortex and the hippocampus and is known to be involved in a variety of normal brain functions including cognition, memory and learning [106].

In the glutamatergic system of the brain (Figure 4) [253, 254], glutamate is synthesized in neurons and stored in synaptic vesicles by the action of vesicular glutamate transporters (VGLUTs). When the presynaptic neuron is stimulated (depolarized), these synaptic vesicles fuse with the plasma membrane and release glutamate to the synaptic cleft, where it exerts its signaling function by binding to and thereby activating glutamate receptors on postsynaptic cells. However, released glutamate immediately needs to be



removed from the synaptic cleft in order to terminate signal transmission and prevent neurotoxic effects from persistent activation, a pathological process known as excitotoxicity. This process is achieved mainly through uptake of glutamate by the high-affinity glutamate transporters (also known as the excitatory amino acid transporters; EAATs) on surrounding astrocytes (EAAT-1 and -2 subtypes) and to a lesser extent by transporters on pre-and postsynaptic neurons (EAAT-3 and -4 subtypes). Within astrocytes, glutamate is enzymatically converted to glutamine by glutamine synthetase. Glutamine is then released and subsequently taken up by the presynaptic terminal, where glutaminase converts it back to glutamate to be packaged into synaptic vesicles and stored for later release. This process is called the glutamate-glutamine cycle.

There are two major classes of mammalian glutamate receptors located at the plasma membrane of neurons, ionotropic and metabotropic [253, 254]. The ionotropic glutamate receptor (iGluR) is a ligand-gated ion channel that allows specific ions such as sodium ( $\text{Na}^+$ ), potassium ( $\text{K}^+$ ) or calcium ( $\text{Ca}^{2+}$ ) to pass across the postsynaptic membrane rapidly upon its binding to glutamate. This flow of ions results in membrane depolarization and the generation of an action potential that is propagated down the processes (dendrites and axons) of the neuron to the next in line. By contrast, the activation of metabotropic glutamate receptors (mGluRs) occurs through several second messenger signaling pathways coupled to G proteins. Therefore, the activation of iGluRs is responsible for fast excitatory synaptic transmission, while mGluRs mediated slower synaptic responses are thought to be involved in the neuromodulatory action of glutamate [255]. The iGluRs can be further divided into three different subtypes according to their pharmacological properties to selective agonists; N-methyl-D-aspartate (NMDA),  $\alpha$ -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid (AMPA), and kainate (KA). Although all iGluRs are equally permeable to monovalent cations,  $\text{Na}^+$  and  $\text{K}^+$ , the NMDA subtype have a particular higher permeability to the divalent cation,  $\text{Ca}^{2+}$ . The mGluRs are currently classified into three distinct groups with a total of eight subtypes based on sequence homology, ligand selectivity, and the second messenger system that is activated upon glutamate binding. In



general, group I mGluRs (mGluR1 and 5) are predominantly coupled to the activation of phospholipase C (PLC) and intracellular calcium signaling which function to increase neuronal excitability, whereas group II (mGluR2 and 3) and group III (mGluR4, 6, 7, and 8) mGluRs are negatively coupled to adenylyl cyclase which function to inhibit neurotransmitter release.

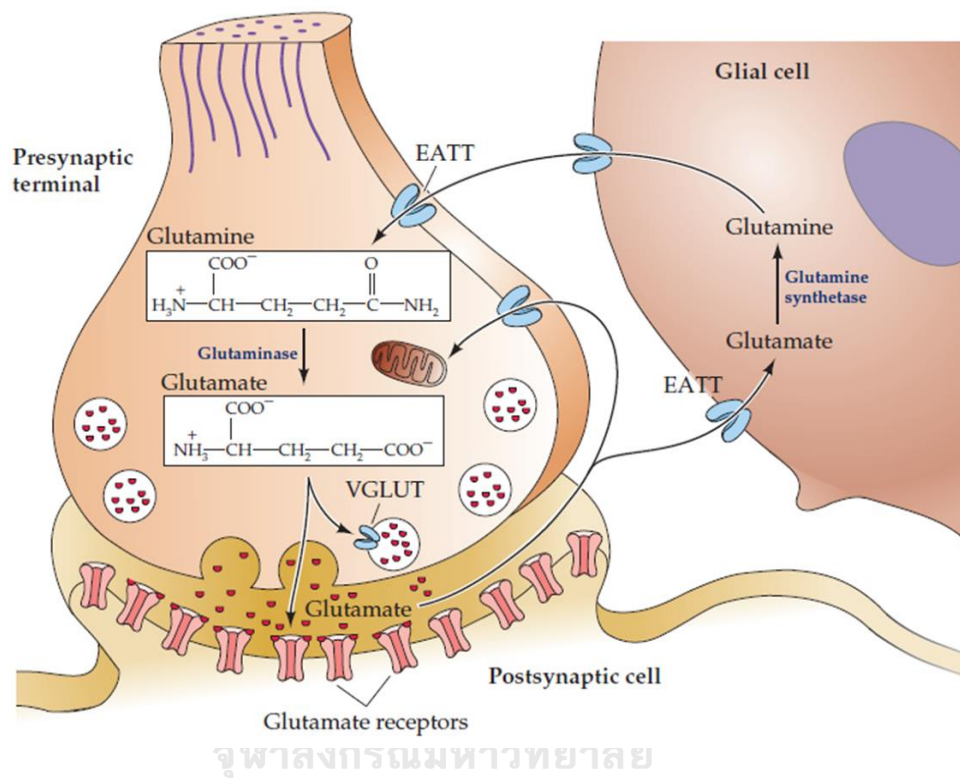


Figure 4: Glutamate synthesis and cycling between neurons and glial cells.

(Image from [253])

## 2.5 Glutamate toxicity and neurodegeneration

Although glutamate is found highly concentrated throughout the brain, an only tiny fraction of this normally presents in the extracellular space (outside and between the cells) which the intracellular glutamate levels are several thousand-fold greater than those in extracellular. Since glutamate receptors are present in most of the neurons and glial cells in the CNS, extracellular glutamate concentrations are tightly controlled to be low by



a number of mechanisms at the synapse to prevent neurotoxic effects from persistent activation [23]. However, perturbations to this regulatory system can have deleterious effects as the excess release of glutamate, which can induce excitotoxicity and contribute to neuronal cell death. The neurotoxicity of glutamate has been implicated in the pathogenesis of a wide range of neurodegenerative diseases (e.g. AD, Parkinson's disease, Huntington's disease, and multiple sclerosis) [20, 21, 34] as well as mood disorders (major depressive disorder and bipolar disorder) [256].

Glutamate-induced neuronal cell death can be mediated by two different pathways, receptor- and non-receptor-dependent (Figure 5). The classical receptor-initiated toxicity pathway, known as excitotoxicity, occurs through excessive stimulation of glutamate receptors, especially the NMDA subtype. Overactivation of these receptors leads to an increase in intracellular  $\text{Ca}^{2+}$  ( $\text{Ca}^{2+}$  influx), which can trigger a cascade of events eventually leading to cell death. These downstream effects include activation of cellular structure-degrading enzymes (e.g. nucleases, proteases and lipases), caspase activation, endoplasmic reticulum (ER) stress, mitochondrial membrane depolarization, and production of toxic oxygen and nitrogen free radicals [257]. In contrast, glutamate cytotoxicity can also be induced by a receptor-independent mechanism involving oxidative stress, often called oxidative glutamate toxicity or oxytosis [40]. In this pathway, high levels of glutamate reduce uptake of cystine, a basic building block of glutathione (GSH), via competitive inhibition or even reversing the action of the cystine/glutamate antiporter (system Xc<sup>-</sup>) [41, 42], resulting in enhanced cellular oxidative stress due to an accumulation of ROS as a consequence of intracellular GSH depletion [258]. Indeed, oxidative glutamate toxicity is also a component of the excitotoxicity cascade [259], in which high intracellular  $\text{Ca}^{2+}$  influx caused by overstimulation of NMDA-type glutamate receptors could induce more ROS production [257]. On the other hand, these ROS could in turn contribute to more release of  $\text{Ca}^{2+}$  from both mitochondria and ER stores into the cytosol [131]. Excessive intracellular ROS levels have detrimental effects on mitochondrial structure and function that eventually lead to neuronal cell death. Several previous



studies have demonstrated that mitochondrial apoptosis inducing factor (AIF) signaling underlies the pathway of programmed cell death induced by oxidative glutamate toxicity in neurons. Several previous studies were consistent with an observation that glutamate-induced apoptosis in HT22 cells is mediated via the caspase-independent pathway, as pan-caspase inhibitor (z-VAD) and specific caspase-3 inhibitor failed to protect against glutamate-induced cell death [43, 260, 261]. The release of pro-apoptotic protein AIF from mitochondria in glutamate-induced oxidative toxicity pathway is involved with the activation of pro-apoptotic protein Bid downstream of 12/15-lipoxygenase (LOX)-dependent lipid peroxidation. Upon release into the cytosol, AIF then translocates to the nucleus, where it induces DNA fragmentation, chromatin condensation, and cell death [43, 44, 260-262]. Activation of signaling molecules in the mitogen-activated protein kinase (MAPK) pathway (e.g. ERK) was also implicated in the mechanism of glutamate-induced oxidative toxicity and appears to be downstream of lipid peroxidation [43, 263].

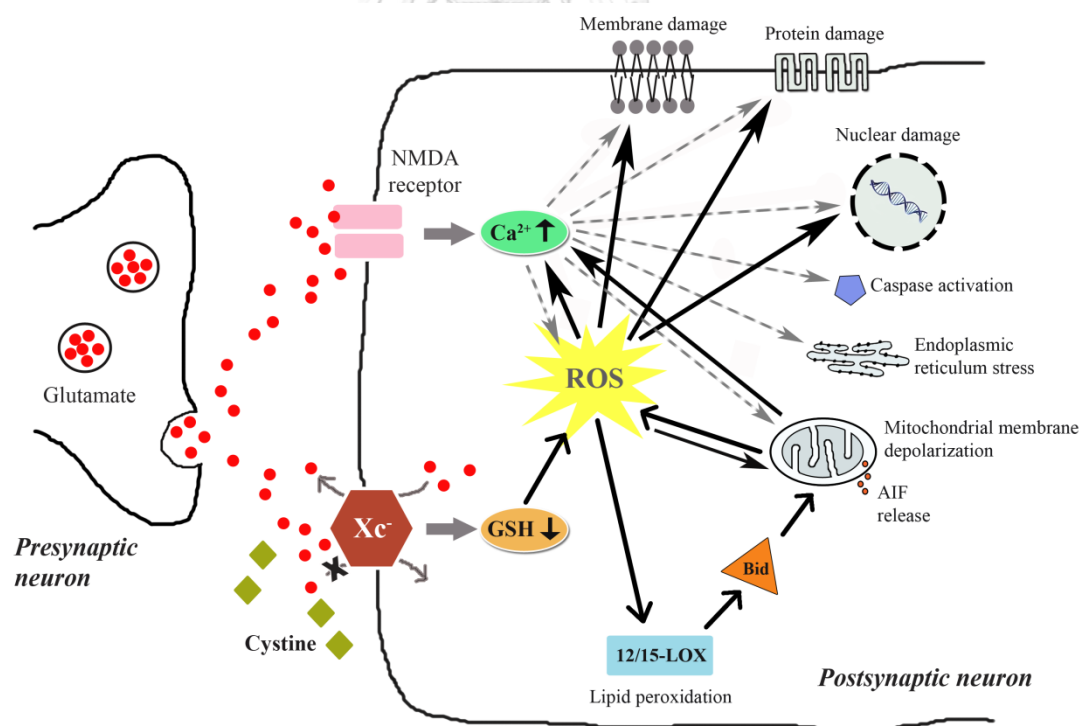


Figure 5: Scheme illustrating the molecular mechanisms of glutamate-induced neurocytotoxicity



## 2.6 Oxidative stress and antioxidant defense system

### 2.6.1 Oxidative stress and reactive oxygen species

Oxidative stress is a widely-used term in biology and medicine, which has been classically defined as an imbalance between oxidants and antioxidants in favor of the oxidants, leading to a disruption of redox signaling and control and/or molecular damage [264]. The oxidants refer to reactive molecules that are capable of causing the process of oxidation to other molecules, often resulting in cell damage and cell death. The majority of reactive molecules that are potentially leading to cellular damage are derived from oxygen, commonly called reactive oxygen species (ROS), which most of them are generated endogenously as byproducts of oxygen metabolism in mitochondria. Oxygen is an essential element for generation of energy in the form of adenosine triphosphate (ATP) to support cellular activities. However, about 1-2% of total oxygen consumed can be converted to short-lived intermediates known as ROS due to the leakage of electrons from complex I and III of mitochondrial electron transport chain during the process of ATP production [265, 266]. The formation of ROS from molecular oxygen is illustrated in Figure 6. In fact, ROS is a collective term used to describe a number of reactive molecules including radicals (e.g. superoxide ( $\text{O}_2^{\cdot -}$ )), and non-radicals ((e.g. hydrogen peroxide ( $\text{H}_2\text{O}_2$ ))) derived from molecular oxygen (Table 2). The radicals are molecules that contain at least one unpaired electron, making them unstable and highly reactive, whereas the non-radicals are specific oxidizing molecules that can be easily converted into radicals. Under physiological condition, these ROS are usually removed or inactivated by the natural antioxidant system of the body.



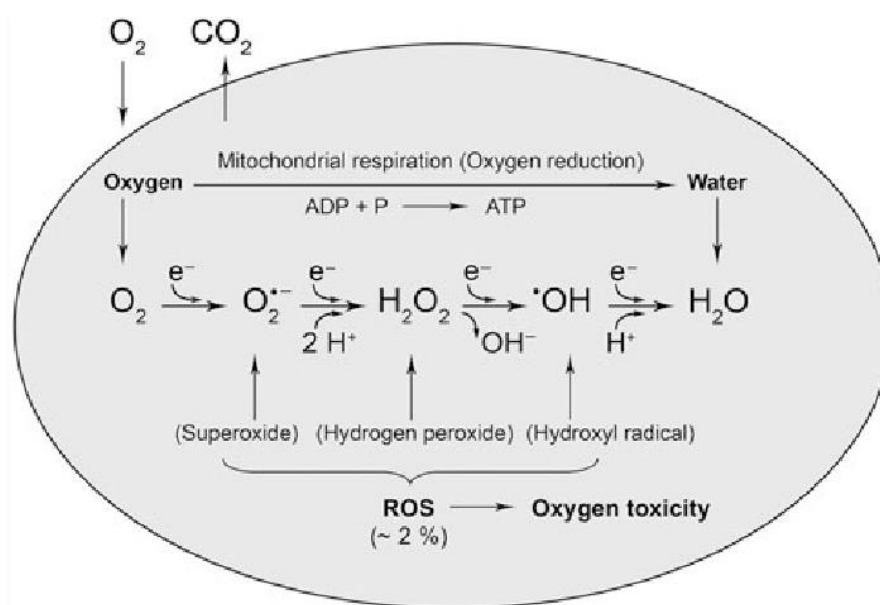


Figure 6: Scheme illustrating the formation of reactive oxygen species (ROS) as byproducts of oxygen metabolism during the generation of ATP in mitochondria.

(Image from [267])

Table 2: Lists of major reactive oxygen species (ROS) produced in the cell.

Radicals		Non-radicals	
Name	Symbol	Name	Symbol
Superoxide	$O_2^{\bullet -}$	Hydrogen peroxide	$H_2O_2$
Hydroxyl	$OH^{\bullet}$	Singlet oxygen	$^1O_2$
Hydroperoxyl	$HO_2^{\bullet}$	Ozone	$O_3$
Alkoxyl	$RO^{\bullet}$	Hypochlorous acid	$HOCl^{\bullet}$
Peroxyl	$RO_2^{\bullet}$	Peroxynitrite	$ONOO^{\bullet}$

Over the past decades, it is now apparent that ROS is not only harmful to the cells. Several lines of evidence have accumulated to support the beneficial role of ROS at a low level as intracellular signaling molecules involved in the regulation of normal physiological processes such as cell growth and differentiation, cell apoptosis, phagocytic



immune response, synaptic plasticity, as well as sperm functioning [268-274]. However, if production of ROS overwhelms the body's natural antioxidant defenses (a state termed oxidative stress), excessive amounts of ROS, in turn, cause potential damage to biomolecules such as lipids, proteins, and nucleic acids, as well as signal transduction of redox-sensitive factors, which ultimately lead to the activation of cell death [145]. ROS and oxidative stress are thought to play an important role in the pathogenesis of many human diseases including cancer, atherosclerosis, diabetes, and neurodegenerative diseases [275, 276].

## 2.6.2 Antioxidant defense mechanisms

### 2.6.2.1 Endogenous antioxidants

Free radicals and other reactive species generated in the body can be naturally removed or prevented them from forming by antioxidant defense mechanisms, which are believed to be necessary for all oxygen-metabolizing cells to live. Antioxidants can be exogenous, consumed from diet and other sources (e.g. vitamins and phytochemicals), or endogenous, produced inside our body. Endogenous antioxidants are known to be important since they serve as the potent first line of defense against ROS through enzymatic inactivation (Figure 7). The components in endogenous defense mechanism can further be classified as enzymatic antioxidants and non-enzymatic antioxidants. Superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) are three of the primary enzymes involved in the direct elimination of active oxygen species,  $O_2^{\bullet -}$  and  $H_2O_2$ . In addition, glutathione peroxidase (GR), glutathione synthetase (GS), gamma-glutamylcysteine synthetase ( $\gamma$ -GCS) and glucose-6-phosphate dehydrogenase (G6PD) are the secondary enzymes that function to maintain a steady supply of metabolic intermediates (non-enzymatic antioxidants) like glutathione (GSH), nicotinamide adenine dinucleotide phosphate (NADP(H)) and glucose 6-phosphate for optimum functioning of the primary antioxidant enzymes. Endogenous non-enzymatic antioxidants also protect cells against



reactive species through other different mechanisms [277]. For example, metal-binding proteins (MBPs) such as albumin, metallothionein, ceruloplasmin, myoglobin, ferritin, transferrin and lactoferrin can bind to pro-oxidant metal ions, such as iron (Fe) and copper (Cu), resulting in prevention of the formation of hydroxyl radicals ( $\text{OH}^\bullet$ ) in the Fenton reaction. Some of MBPs, albumin and metallothionein, can directly scavenge ROS including  $\text{OH}^\bullet$ ,  $\text{O}_2^{\bullet -}$ , peroxynitrite ( $\text{ONOO}^-$ ), and hypochlorous acid ( $\text{HOCl}$ ) [278]. Such MBP like myoglobin is a scavenger of nitric oxide (NO) [279]. Coenzyme  $\text{Q}_{10}$  ( $\text{CoQ}_{10}$  also known as ubiquinone) is an endogenously produced antioxidant compound that plays a substantial role in energy production by acting as a mobile electron carrier in the electron transport chain. The antioxidant property of  $\text{CoQ}_{10}$  is demonstrated in its fully reduced form, ubiquinol ( $\text{CoQH}_2$ ), which has the ability to act as a powerful fat-soluble free radical scavenger by affecting the initiation and propagation of lipid peroxidation, thereby preventing against lipid radicals produced in biological membranes [280].

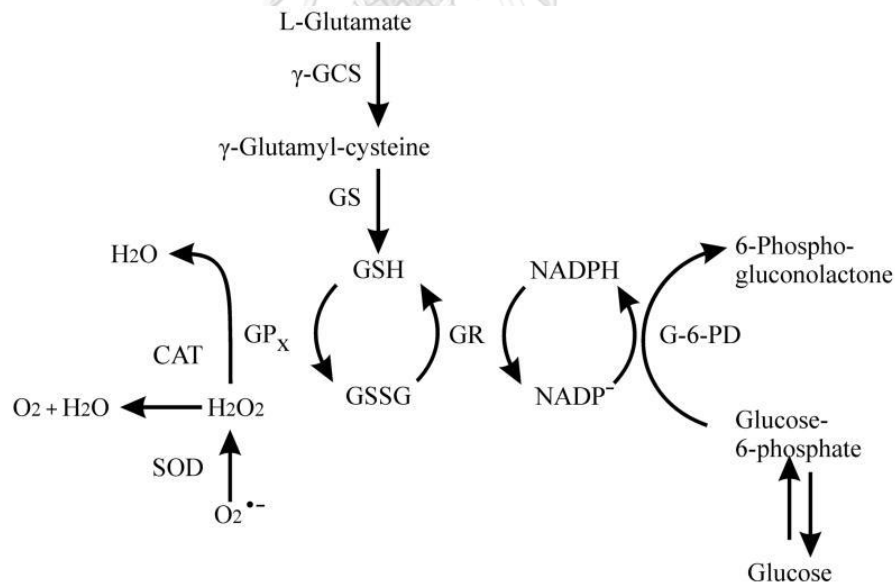


Figure 7: Scheme illustrating the endogenous antioxidant defense mechanism against ROS. The superoxide dismutase (SOD) converts superoxide radicals ( $\text{O}_2^{\bullet -}$ ) into hydrogen peroxide ( $\text{H}_2\text{O}_2$ ), which subsequently be reduced to water ( $\text{H}_2\text{O}$ ) and oxygen ( $\text{O}_2$ ) by glutathione peroxidase (GPx), or to only  $\text{H}_2\text{O}$  by catalase (CAT). The action of GPx also requires several



secondary enzymes including glutathione peroxidase (GR), glutathione synthetase (GS), gamma-glutamylcysteine synthetase ( $\gamma$ -GCS) and glucose-6-phosphate dehydrogenase (G6PD), as well as antioxidant cofactors including glutathione (GSH), nicotinamide adenine dinucleotide phosphate (NADP(H)) and glucose 6-phosphate to function at high efficiency. GSH is synthesized in two steps from three amino acid precursors; glutamate, cysteine, and glycine. (Image from [281])

### 2.6.2.2 The Nrf2/ARE signaling pathway

Induction of the Nrf2/ARE signaling pathway is a major mechanism in the cellular protection against oxidative or electrophilic stress by controlling the expression of genes whose protein products are involved in the detoxification and elimination of reactive oxidants and electrophilic agents through conjugative reactions and enhancing cellular antioxidant capacity. This pathway is mediated by the activation of the nuclear factor (erythroid-derived 2)-related factor 2 (Nrf2, also known as NFE2L2), a transcription factor that regulates the expression of many antioxidant and detoxifying enzymes/proteins by binding to a cis-acting regulatory enhancer region termed antioxidant response element (ARE) in the promoter of genes encoding for those proteins. The core consensus sequence of functional ARE was defined as 5'-RTGACnnnGC-3', where n represents any nucleotide and R represents A or G [282]. Some of the well-characterized ARE-driven cytoprotective genes controlled by Nrf2 include system Xc<sup>-</sup>, heme oxygenase 1 (HO-1), NAD(P)H:quinone oxidoreductase 1 (NQO1), superoxide dismutase (SOD), thioredoxin (Trx), glutathione S-transferase (GST) and the rate-limiting enzymes of glutathione synthesis consisting of catalytic (GCLC) and modifier (GCLM) subunits [283, 284], as well as Nrf2 itself [285].

Figure 8 shows the activation of Nrf2 pathway in response to stress signals which leads to upregulation of genes under Nrf2 control. Under normal physiological conditions, most of the transcription factor Nrf2 is retained in the cytoplasm before being rapidly degraded by the ubiquitin-proteasome pathway via its interaction with the Kelch-like ECH-



associated protein 1 (Keap1) homodimer and the scaffolding protein E3 ubiquitin ligase Cullin 3 (Cul3) [286]. However, in conditions of oxidative or electrophilic stress, the critical cysteine residues in Keap1 are disrupted, preventing the ubiquitination of Nrf2 by Cul3. This leads to the accumulation of free Nrf2 in the cytoplasm and subsequently translocation into the nucleus where it forms a heterodimer with a small Maf protein (Maf). The Nrf2-Maf complex then binds to the ARE sequence in the upstream promoter regions of several Nrf2-controlled genes and initiates their transcription [284]. Currently, up-regulation of the Nrf2/ARE signaling pathway such as overexpression of Nrf2 has been suggested as a potential therapeutic target for the treatment of various neurodegenerative disorders including AD [283, 287].

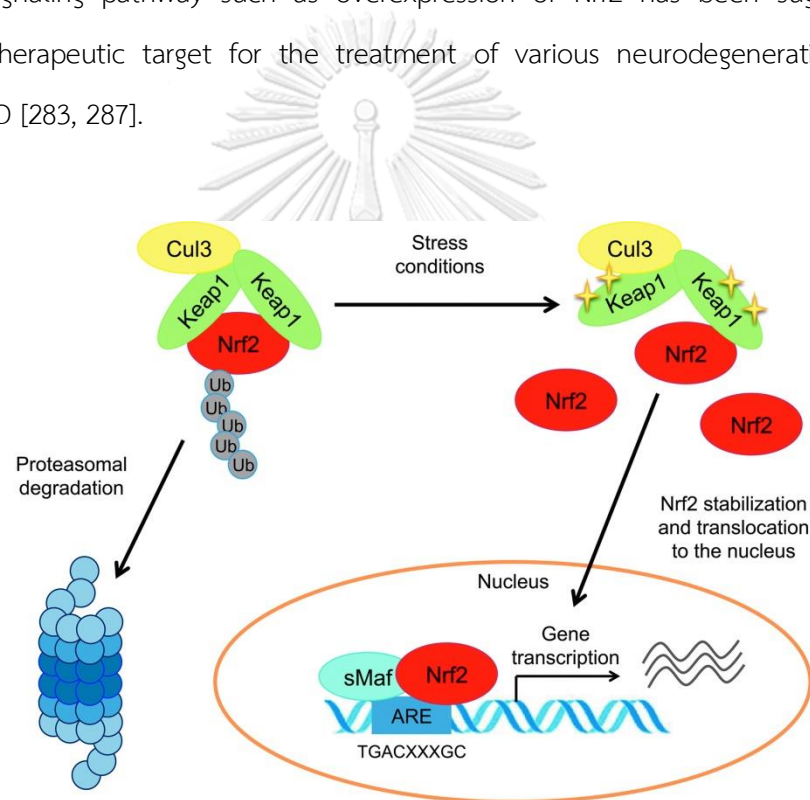


Figure 8: Schematic representation of the Nrf2/ARE signaling pathway under oxidative/electrophilic stress conditions.

(Image from [284])



### 2.6.2.3 The cystine/glutamate antiporter system $Xc^-$

System  $Xc^-$  is a membrane-bound,  $Cl^-$ -dependent,  $Na^+$ -independent antiporter that typically mediates the 1:1 exchange of extracellular cystine and intracellular glutamate across the cellular plasma membrane [288]. The amino acid cystine is an oxidized form of cysteine, a building block of glutathione (GSH), which is a major cellular antioxidant responsible for the maintenance of intracellular redox homeostasis [289]. Under physiologic conditions, this system serves as a rate-limiting step in providing cysteine into cells for the synthesis of GSH in brain cells as well as a source of non-vesicular glutamate release. However, an increased efflux of intracellular glutamate through this exchanger has a potential to contribute to excitatory signaling and/or excitotoxic pathology [290]. Additionally, an excessive extracellular glutamate concentration can also induce inhibition of this transport system (known as oxidative glutamate toxicity), resulting in GSH deprivation, oxidative stress, and eventual cell death [258].

Structurally, system  $Xc^-$  is composed of a catalytic light-chain subunit xCT, which confers transport activity with substrate specificity, and a regulatory heavy-chain subunit 4F2, which common to all amino acid transporters [291]. The subunit xCT is inducible and belongs to a family of proteins involved in the defense against xenobiotic and oxidative stress, which the *SLC7A11* gene encoded for this subunit contains an antioxidant-response element (ARE) in the promoter region [288]. The induction of xCT mRNA expression by electrophilic agents is mediated through the Nrf2/ARE pathway [292]. Given an essential dual role of this system in maintaining a critical intracellular antioxidant GSH and in non-vesicular glutamate release, there has been suggested its potential involvement in the pathogenesis of a wide array of diseases including various neurodegenerative disorders [288, 290, 293]. Interestingly, overexpression of the system  $Xc^-$  subunit xCT and the glutamate transporter EAAT3 have been reported to cooperatively protect neuronal cells from glutamate toxicity by reducing glutamate-induced GSH depletion [258], suggesting that system  $Xc^-$  could be a promising therapeutic target for neurodegenerative diseases.



#### 2.6.2.4 NAD(P)H:Quinone Oxidoreductase 1

NAD(P)H:quinone oxidoreductase 1 (NQO1, previously known as DT-diaphorase) is a cytosolic FAD-containing protein that exists as a homodimer and acts as an antioxidant and detoxification enzyme in the cell [294, 295]. It is encoded by the *NQO1* gene containing an antioxidant-response element (ARE) in the promoter region which renders this protein highly inducible under a variety of stress responses including oxidative stress and strongly upregulated by the Nrf2/ARE pathway [296]. NQO1 utilizes either NADH or NADPH as reducing cofactors and catalyzes the two-electron reduction of a broad range of substrates, most notably quinones. This reaction ensures complete oxidation of the substrate without formation of the highly reactive semiquinones, superoxide radicals ( $O_2^{\cdot -}$ ), as well as other reactive species that are deleterious to cells [297]. This enzyme also has ability to restore antioxidant activity of  $\alpha$ -tocopherol quinone, a product of free radical attack of  $\alpha$ -tocopherol (vitamin E) that is devoid of direct antioxidant capability [298]. Moreover, previous studies have revealed other recognized cytoprotective roles of NQO1 that are unrelated to its enzymatic activities such as protection of proteins (e.g. tumor suppressor p53) against ubiquitin-independent 20S proteasomal degradation [299, 300].

#### 2.6.2.5 Glutamate-cysteine ligase modifier subunit

Glutamate-cysteine ligase modifier subunit (GCLM) is a light (31 kDa) modulatory subunit of the heterodimeric enzyme gamma-glutamylcysteine synthetase ( $\gamma$ -GCS) involved in the formation of gamma-glutamylcysteine ( $\gamma$ -GC), which is the first and rate-limiting step of GSH synthesis. While, the catalytic subunit (GCLC) of  $\gamma$ -GCS enzyme binds glutamate and cysteine and catalyzes their ligation, GCLM regulates the binding affinity of the GCLC to its substrates or inhibitors [301]. GCLM is encoded by the *GCLM* gene that contains an antioxidant-response element (ARE) in the promoter region, thus enabling its upregulation by Nrf2 transcription factor in response to oxidative stress [302].



### 2.6.2.6 Excitatory amino acid transporter 3

Excitatory amino acid transporter 3 (EAAT3) is high-affinity amino acid transporter subtype 3 that are dependent on an electrochemical gradient of  $\text{Na}^+$  ions. EAAT3, also termed as EAAC1 (excitatory amino acid carrier 1) in non-human species, is encoded by the *SLC1A1* gene and ubiquitously expressed throughout the brain. Activation of the Nrf2/ARE pathway by oxidative stress also promotes the transcription of this transporter, whose promoter region of encoded gene contains an antioxidant-response element (ARE) [303]. In general, EAAT3 mediates the intracellular uptake of amino acid molecules, predominantly glutamate and cysteine, together with three  $\text{Na}^+$  and one  $\text{H}^+$ , in parallel with the counter-transport of one  $\text{K}^+$  ion [304, 305]. Therefore, EAAT3 plays a crucial role in glutamatergic neurotransmission by regulating the efficient clearance of glutamate from the extracellular space, which is essential for terminating the postsynaptic action of glutamate. Moreover, it was suggested to have a modulatory role in neurotransmission by regulating the AMPA-type glutamate receptor synaptic localization and stability [306]. EAAT3 is also important in supplying glutamate as a precursor for gamma-aminobutyric acid (GABA) synthesis in GABAergic neurons. Importantly, EAAT3 is the major route of neuronal cysteine uptake, which is the rate-limiting factor for synthesis of GSH.



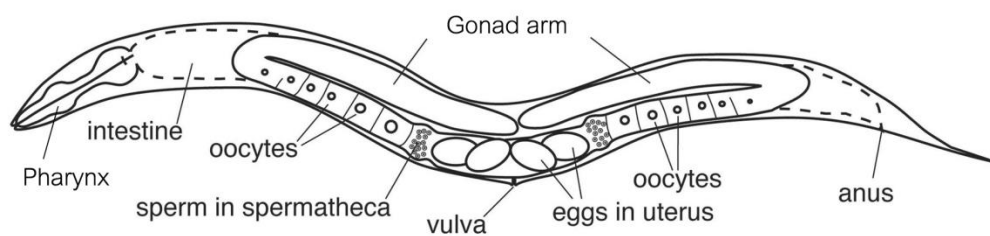
## 2.7 *Caenorhabditis elegans* model organism

*Caenorhabditis elegans* (*C. elegans*) is a small (adult size about 1 mm in length and 80  $\mu\text{m}$  in diameter), transparent, free-living nematode (roundworm) which naturally lives in the soil and rotting vegetation in many parts of the world, where it survives by feeding on microbes, primarily bacteria, through the large pump-like organ called the pharynx. *C. elegans* has a simple anatomy with about 1,000 somatic cells organized in a small number of tissues and internal organs. Like all nematodes, the basic anatomy of *C. elegans* includes a digestive system, a reproductive system, a nervous system, a muscle system, and an excretory system, while they have neither a circulatory nor a respiratory



system. *C. elegans* occurs in two natural sexes, a hermaphrodite (XX genotype) and a male (XO genotype), which differ extensively in anatomy, physiology, and behavior. Figure 9 shows the basic anatomy of the adult *C. elegans* hermaphrodite and male. The hermaphrodite produces a limited number of sperm during late larval development before switching to begin oogenesis in the adult stage, therefore it can be regarded as a female with stored sperm that can reproduce either by self-fertilization, using its own sperm, or by cross-fertilization after mating, using sperm contributed by a male [307]. Reproduction by self-fertilization allows for the generation of genetically identical progeny, while male mating facilitates genetic crosses and producing heterozygous offspring. Among the self-progeny of hermaphrodites, the dominant sexual form of the offspring is the hermaphrodite, while males normally arise at a low frequency (0.1%) due to spontaneous nondisjunction of the X chromosome. However, males can also be produced at a higher frequency (up to 50%) through mating.

#### Hermaphrodite (XX)



#### Male (XO)

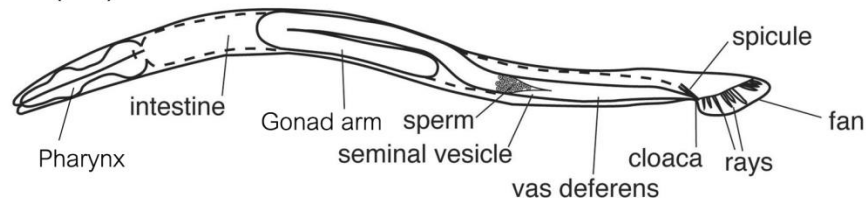


Figure 9: Schematic drawing of anatomical structures of adult *C. elegans*.

Hermaphrodite (top) and Male (bottom) (Image from ©Wormatlas)



The life cycle of *C. elegans*, like that of other nematodes, is relatively short and temperature-sensitive (within 2-3 days at 25°C) comprised of the embryonic stage (fertilized egg), four larval stages (designated as L1-L4) and adulthood (adult) (Figure 10 and 11). Following completion of *in utero* and *ex utero* embryonic development, the *C. elegans* first stage (L1) larvae hatch from the eggshell and begin feeding. Under favorable growth conditions (e.g. abundant food, low temperature, and low population density), the larvae proceed through three more larval stages (L2, L3, and L4) before becoming reproductive adults. The mature adults can live for an additional 15-20 days, which the reproductive period of a hermaphrodite generally is the first 4–5 days of adulthood. However, if during development, the L1 larvae encounter harsh environments that are unfavorable for further growth (e.g. limited food, high temperature, and high population density), they will enter into a physiologically specialized larval stage called dauer, an alternative form of third larval stage (L3) that is developmentally arrested. The dauer larvae are highly resistant to environmental stress and can survive for several months without feeding. Once better growth conditions are available, they exit the dauer larval stage and resume normal reproductive development into the fourth stage (L4) larvae, and subsequently, into adults with a normal lifespan (about 3 weeks at 20°C [308]).



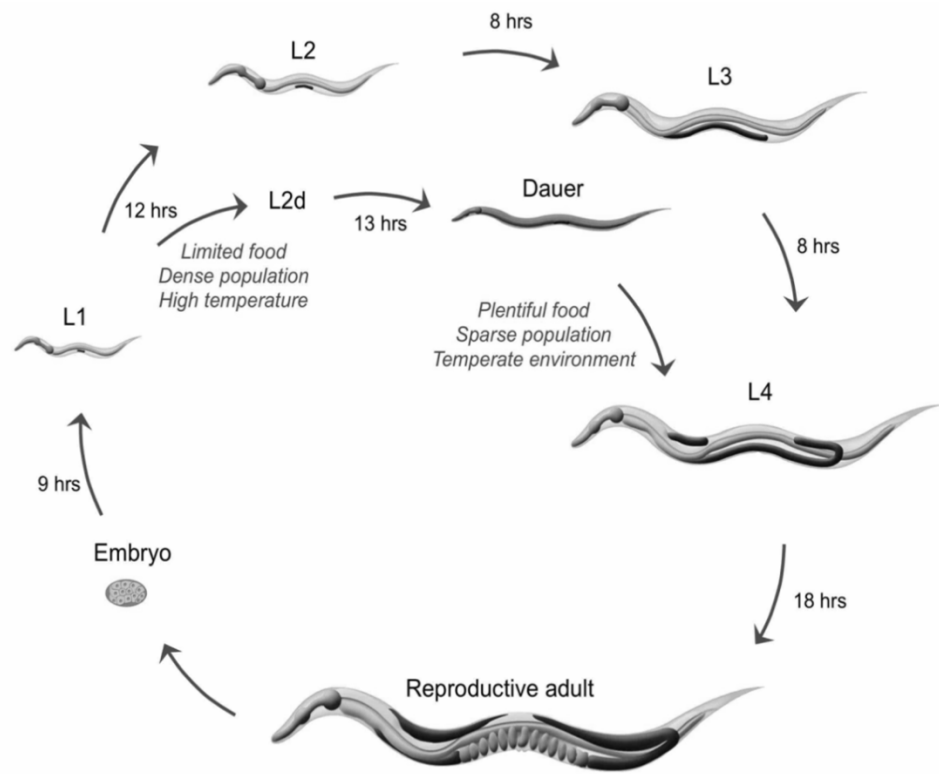


Figure 10: Life cycle of *C. elegans* at 25°C.

(Image from ©Wormatlas)



Figure 11: Images of the eggs, the four larval stages (L1-L4), and the adulthood of *C. elegans* observed by microscopy at 50X magnification.

(Image from [309])



After being first introduced by Sydney Brenner in the 1960s [310, 311], nowadays *C. elegans* is an important model organism widely used in many research areas, either to study the basic genetic and molecular mechanisms underlying the disease pathogenesis or to serve as a drug screening model in drug discovery related to diseases. A large number of human diseases that have been investigated using *C. elegans* include diabetes, obesity, cancer, depression, aging, Alzheimer's disease and other neurodegenerative diseases [309, 312]. *C. elegans* has a number of characteristic features that make it a powerful *in vivo* model system for biological research, particularly in the field of aging [313-315]. First of all, *C. elegans* is easy and inexpensive to cultivate in the laboratory conditions. It can also be stored in a frozen stock for several months with over 50% thawing survival. Moreover, *C. elegans* has a relatively short lifespan with a rapid life cycle and high reproductive capacity. The worm also has a transparent body that allows for the real-time visualization of intracellular structures of interest. Lastly, its genome is fully sequenced and well annotated that enables the ease of genetic manipulation and analysis. In fact, *C. elegans* was the first multicellular organism with a complete genome sequence (approximately 19,000 genes) [316]. The biology of aging in *C. elegans* may also be applicable and relevant to human aging. Normal aging in *C. elegans* is accompanied by increased wrinkling in the cuticle, declines in mobility and reproductive capacity as well as the ability to sense and respond to environmental stimuli [317]. Figure 12 represents aging in *C. elegans* that the older worm showed the more wrinkled appearance of the cuticle than the young worm.



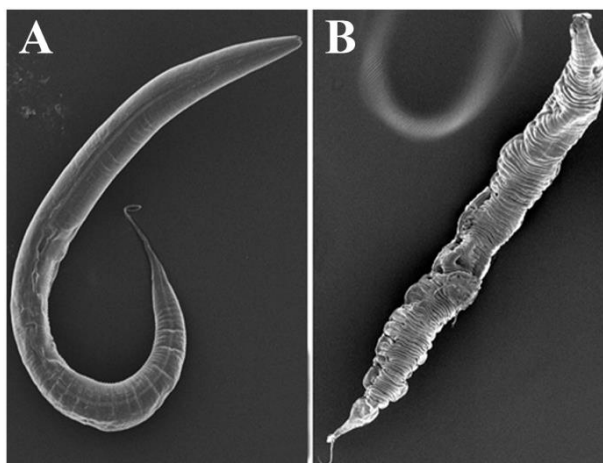


Figure 12: Scanning electron microscopy images of young (A) and old (B) *C. elegans* adults.

(Image from ©The Ghazi Laboratory)

## 2.8 Plants used in the study

### 2.8.1 *Morinda citrifolia* Linn.

*Morinda citrifolia* Linn. is a small evergreen tree in the family Rubiaceae which can grow up to 10 m tall. The fruit has oval-shaped about 5-10 cm long with irregular yellowish or whitish color and uneven surface covered by polygonal-shaped sections. This plant is native to Southeast Asia, Australia, and the islands of Polynesia (e.g. Hawaii) and popularly known as noni (Hawaiian) or Indian mulberry (English) or Yor-Ban (Thai) [318]. *M. citrifolia* has a long history as a medicinal plant that has been traditionally used for thousands of years to treat a variety of health problems such as headache, fever, cough, pain, burns, wounds, inflammation, arthritis, gingivitis, diarrhoea, dysentery, tuberculosis, asthma, hypertension, diabetes, cancer, as well as parasitic, viral, and bacterial infections [319]. Interestingly, it has also been used as an ingredient in a traditional formula for longevity in Thailand [320]. In addition, *M. citrifolia* have reported a broad range of therapeutic effects, including antibacterial, antiviral, antifungal, analgesic, vasodilatory, antioxidant, anti-inflammatory, antithrombotic, anti-tubercular, anti-arthritic, anti-diabetic, anti-cancer,



immune enhancing, anxiolytic, sedative, anti-AChE, neuroprotective and memory enhancing activities [321-327]. Until now, approximately 200 phytochemical compounds have been already identified in this plant, which the majority of them are phenolic compounds, organic acids and alkaloids. Other components include polysaccharides, fatty acids, lignans, carotenoids, iridoids, triterpenoids, coumarins, and phytosterols [318, 319, 327].

### 2.8.2 *Caesalpinia mimosoides* Lamk.

*Caesalpinia mimosoides* Lamk, commonly known as Mimosa Thorn (English) or Cha-rueat or Phak puya (Thai), is a small spiny climbing shrub in the family Fabaceae that grows up to one meter tall. Its branches armed with straight prickles with many bright yellow flowers. This plant is native to the Indian subcontinent and Southeast Asia including the northern part of Thailand. *C. mimosoides* is one of the important traditional folk medicinal plants used in the treatment of boils, epilepsy, arthritis, wounds and skin diseases. In Thailand, young sprouts with flowers and leaf are locally consumed as fresh vegetables as they are believed to help to relieve dizziness and flatulence [328]. Moreover, this plant has been reported to exhibit strong antioxidant, antimicrobial, anti-inflammatory, wound healing, anti-cancer, anti-AChE, neurite outgrowth and neuroprotective activities [328-334]. Previous chemical investigations revealed the presence of two major polyphenols, gallic acid and quercetin [328, 330, 334], as well as terpenoids [331, 335] in this plant species.

### 2.8.3 *Murdannia loriformis* (Hassk.) Rolla Rao et Kammathy

*Murdannia loriformis* (Hassk.) Rolla Rao et Kammathy is a perennial herb about 10 cm high in the family Commelinaceae. The leaf is simple, glabrous, and narrow up to 20 cm long. *M. loriformis* originated in Southern China but also found in abundant in



Southeast Asia including Philippines, Vietnam, and Thailand. It is commonly known as Beijing grass (English), Niu e Cao (Chinese), or Ya Pak-king (Thai). This plant has long been used in traditional Chinese medicine and also in Thai folk medicine for longevity [320], and as a remedy for various kinds of health problems such as hypertension, inflamed wounds, cough, fever, cold and respiratory tract complaints. Importantly, it has been extensively used to treat different types of cancer including those of white blood cells, liver, stomach, intestines, uterus and breast [336, 337]. Previous scientific reports have confirmed the anti-mutagenicity, antioxidant, gastroprotective, anti-inflammatory, analgesic, antipyretic and immunomodulatory activities of *M. loriformis* [338-344]. However, it is surprising that, to date, there is only one compound, a cytotoxic glycosphingolipid that helps reduce cancer growth, has reported in this plant species [341].

#### 2.8.4 *Acanthus ebracteatus* Vahl.

*Acanthus ebracteatus* Vahl., commonly known as Sea Holly (English), Jeruju hitam (Malay) or Nguag-plaa-moa (Thai), is a medicinal mangrove plant in the family Acanthaceae and is widely distributed in Southeast Asia, including China, Australia, and Thailand. It is a sprawling shrubby plant up to 2 m tall with dark green leaf that is stiff and deeply lobed with sharp spines at the end of each deep lobe resembles those of holly [345, 346]. All parts of this plant have been used historically for a variety of medicinal purposes, such as hair root nourishment, reduction of cough and fever, expelling intestinal worms, expulsion of kidney stones, relief of rheumatoid arthritis pain and inflammation, and treatment of hypertension, cancer, boils, skin diseases such as rash, chronic wounds and snakebites [337, 347-350]. Interestingly, *A. ebracteatus* is also used as an important ingredient in traditional Thai longevity and neurotonic remedies for improving brain and body functions [351]. Previous chemical investigations on *A. ebracteatus* revealed that the majority of previously reported phytochemical components in this plant are polyphenolic in structure



[348, 352], which is generally known to possess strong antioxidant and anti-inflammatory properties.

#### 2.8.5 *Streblus asper* Lour.

*Streblus asper* Lour. is a well-known medicinal plant that belongs to family Moraceae and distributes mainly over the region of Southeast Asia including Thailand. This plant is known by several common names such as Shakhotaka (Sanskrit), Siora (Hindi), Kesinai (Malay), Siamese rough bush or Tooth brush tree (English), and Khoi (Thai). It is a medium-sized evergreen tree about 5-15 m high with pubescent branchlets and 2–4 inches long, irregularly toothed and rigid leaf [353]. Different parts of the plant have been traditionally used for various medicinal purposes such as treatment of fever, toothache, filariasis, leprosy, snakebite, diarrhoea, piles, epilepsy, epistaxis, heart disease, urinary tract complaints, stomachache, obesity, skin diseases, wounds, and cancer [353, 354]. It has also been used as an ingredient in a traditional Thai formula for longevity [355]. Studies on the crude plant extract as well as the isolated compounds have demonstrated that *S. asper* exhibits various pharmacological properties including antioxidant, anticancer [356], antimicrobial [357], antimalarial [358], anti-filarial [359], anti-inflammation [360], and anti-hepatitis B activities [361]. Previous studies on the phytochemical constituents of *S. asper* have been shown that this plant contains a large number of cardiac glycosides [353]. Other compounds identified from this plant include lignans, flavonoids, triterpenoids, and alkaloids [362-364].



## 2.9 Acid-Base extraction

Acid-Base extraction is a procedure using sequential liquid-liquid extraction to separate a mixture of organic compounds based on their acid-base properties and their relative solubilities in two different immiscible liquids, usually water and an organic solvent. It is commonly used in organic chemistry in order to isolate and purify a crude mixture of organic compounds obtained from natural product extracts into its acidic, basic, and neutral (non-acidic and non-basic) components. In organic chemistry, acids contain the carboxylic ( $\text{-COOH}$ ) and the phenolic ( $\text{Ar-OH}$ ) functional groups. Bases contain at least one nitrogen atom and are commonly called amine group ( $\text{-NH}_2$ ). The fundamental theory behind this technique is that an organic compound to be extracted is chemically converted to an ionic form which is then soluble in an aqueous layer and can be extracted later, whereas other non-ionic organic compounds in the mixture will remain dissolved in the organic solvent layer. Figure 13 details the acid-base extraction process flow for the separation of acidic, basic and neutral compounds present in the mixture. When the acidic or basic organic compounds are treated respectively with aqueous base or acid, it will cause an acid-base reaction that produces an ionic salt. The ionic salt is soluble in water but insoluble in many less polar organic solvents. Once separated, the acidic or basic organic compounds can be recovered from the aqueous solution by neutralization that reverts the salt back to its parent organic molecule, which is no longer soluble in water and can then be isolated into an organic solvent. The neutral organic compound has neither an acidic nor a basic functional group, so they will remain dissolved in the organic layer, no matter which acids or bases is added.



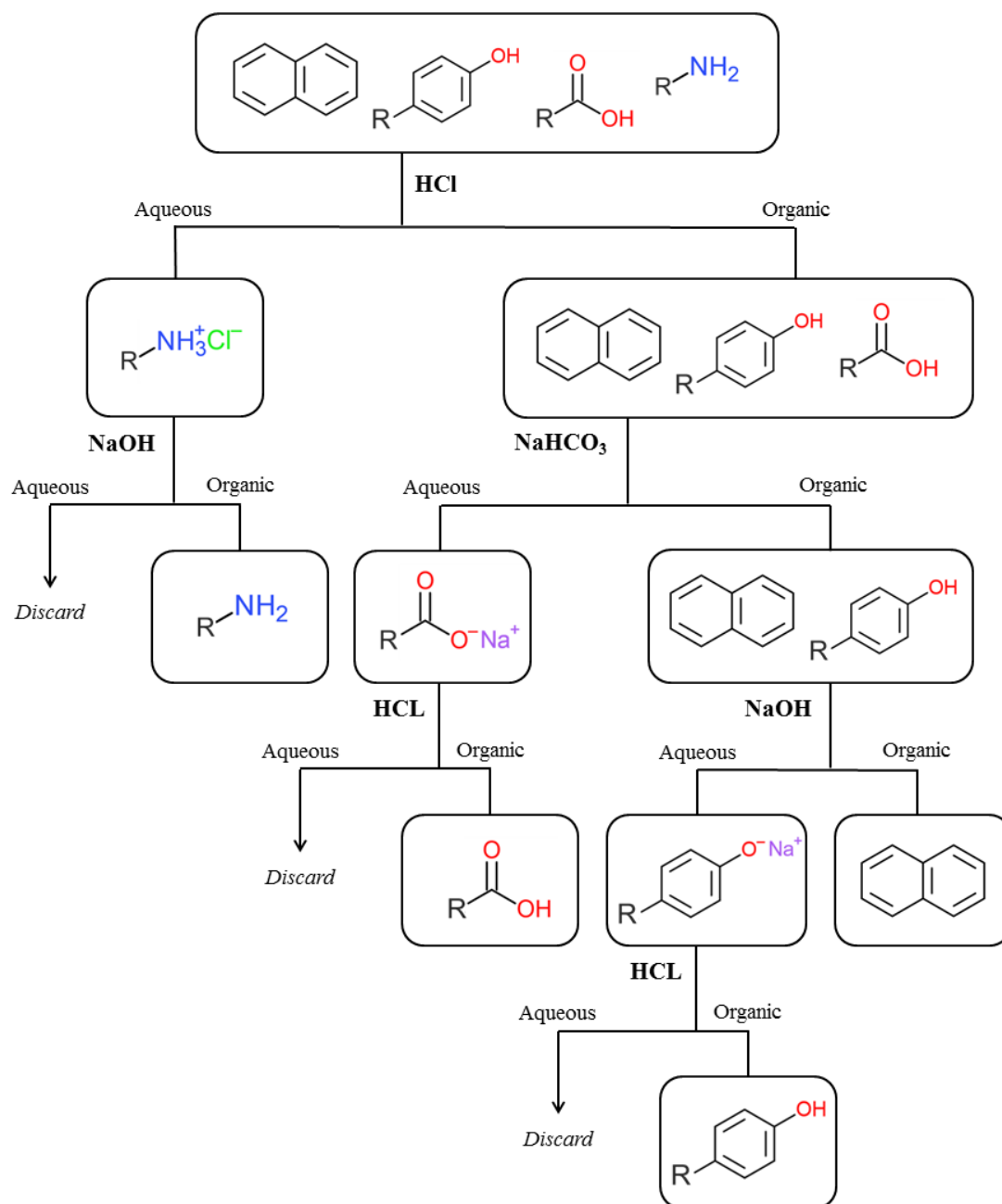


Figure 13: Flowchart of Acid-Base extraction.

Acid-Base extraction for the separation of a four component mixture that contains organic acid (carboxylic acid), weak organic acid (phenolic), organic base (amine), and organic neutral (aromatic hydrocarbon, aliphatic hydrocarbon, alcohol, ester, amide, ether, etc.).



## CHAPTER III

### MATERIALS AND METHODS

#### 3.1 Materials

##### 3.1.1 Chemicals and reagents

Name	Company, Country
2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS)	Sigma-Aldrich, USA
Acetylcholinesterase from electric eel (EC 3.1.1.7, type V-S)	Sigma-Aldrich, USA
30% Acrylamide/Bis solution, 37:5:1	Bio-Rad, USA
Agarose	Merck, Germany
Agar bacteriological (Agar No. 1)	Thermo Scientific, USA
Aluminum chloride ( $\text{AlCl}_3$ )	Sigma-Aldrich, USA
Ammonium persulfate (APS)	Merck, Germany
Antifade mountant	Thermo Scientific, USA
Blotting-grade blocker	Bio-Rad, USA
Bovine serum albumin (BSA), Fraction V	GE Healthcare, USA
Bradford reagent	Bio-Rad, USA
Chloroform	Sigma-Aldrich, USA
Curcumin	Sigma-Aldrich, USA



CytoTox 96 <sup>®</sup> non-radioactive cytotoxicity assay	Promega, USA
4',6-diamidino-2-phenylindole (DAPI)	Molecular Probes, USA
1,4-Dioxane	Merck, Germany
Diethyl pyrocarbonate (DEPC)	Sigma-Aldrich, USA
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich, USA
Dulbecco's modified Eagle's medium (DMEM)	Sigma-Aldrich, USA
DNA Ladder 100 bp	Thermo Scientific, USA
2,2-Diphenyl-1-picrylhydrazyl (DPPH)	Sigma-Aldrich, USA
Dithiothreitol (DTT)	Merck, Germany
ECL Select Western Blotting detection reagent	GE Healthcare, USA
Ethylenediaminetetraacetic acid (EDTA)	Sigma-Aldrich, USA
Ethanol	RCI Labscan, Thailand
Ethyl acetate	RCI Labscan, Thailand
Fast Blue B salt	Sigma-Aldrich, USA
Fetal bovine serum (FBS)	Sigma-Aldrich, USA
FITC Annexin V Apoptosis Detection Kit with PI	BioLegend, USA
Folin-Ciocalteu phenol reagent	Sigma-Aldrich, USA
Galantamine	Sigma-Aldrich, USA
Gallic acid	TCI America, USA



GBX Developer/Fixer	Kodak, USA
Glycine	GE Healthcare, USA
Goat anti-rabbit IgG (H+L), CF™ 555 antibody	Sigma-Aldrich, USA
Goat anti-rabbit IgG, HRP-linked antibody	Cell Signaling Technology, USA
2', 7'-dichlorodihydrofluorescein diacetate (H <sub>2</sub> DCFDA)	Molecular Probes, USA
Hank's balanced salt solution (HBSS)	Gibco, USA
Hexane	RCI Labscan, Thailand
Hydrochloric acid, 37%	Merck, Germany
Hydrogen peroxide (H <sub>2</sub> O <sub>2</sub> ; 30%)	Merck, Germany
Isopropanol	Sigma-Aldrich, USA
L-ascorbic acid	Calbiochem, USA
L-glutamic acid	Sigma-Aldrich, USA
Mouse anti-AIF antibody	Cell Signaling Technology, USA
Mouse anti-β-actin antibody	Cell Signaling Technology, USA
Mouse anti-EAAT3 antibody	Abcam, UK
Mouse anti-Nrf2 antibody	Santa Cruz Biotechnology, USA



Mouse anti-Lamin B1 antibody	Cell Signaling Technology, USA
Mueller-Hinton broth	Himedia laboratories, India
1-Naphthyl acetate	Sigma-Aldrich, USA
Nonidet <sup>®</sup> P 40 Substitute (NP-40)	PanReac AppliChem, Germany
NE-PER <sup>®</sup> nuclear and cytoplasmic extraction reagents	Thermo Scientific, USA
Quercetin	Sigma-Aldrich, USA
Methanol	RCI Labscan, Thailand
3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide (MTT)	Biobasic, Canada
Oligo(dT)17 Primer	Bioneer, South Korea
Paraformaldehyde	Sigma-Aldrich, USA
Penicillin/Streptomycin solution	Gibco, USA
Peptone	Thermo Scientific, USA
Phenylmethylsulfonyl fluoride (PMSF)	Sigma-Aldrich, USA
Phosphate buffered saline (PBS)	Hyclone, USA
Potassium carbonate ( $K_2CO_3$ )	Merck, Germany
Potassium persulphate ( $K_2S_2O_8$ )	Sigma-Aldrich, USA
Primers	Bioneer, South Korea





Propanol	RCI Labscan, Thailand
Protein ladder	Thermo Scientific, USA
PVDF membrane	GE Healthcare, USA
qPCR PreMix	Bioneer, South Korea
RT PreMix	Bioneer, South Korea
Silica gel F <sub>254</sub> TLC plates	Merck, Germany
Sodium acetate (NaOAc)	Sigma-Aldrich, USA
Sodium carbonate (Na <sub>2</sub> CO <sub>3</sub> )	Merck, Germany
Sodium chloride (NaCl)	Merck, Germany
Sodium dodecyl sulfate (SDS)	Ajax Finechem, Australia
Sodium hydroxide (NaOH)	Merck, Germany
Sodium sulfate (Na <sub>2</sub> SO <sub>4</sub> )	Merck, Germany
Tetramethylethylenediamine (TEMED)	Merck, Germany
Tris base	Vivantis Technologies, Malaysia
Tris-HCl	Sigma-Aldrich, USA
Trizol reagent	Invitrogen, USA
Triton X-100	Merck, Germany
Trypan Blue Solution, 0.4%	Gibco, USA



Trypsin-EDTA	Hyclone, USA
Tryptone	Thermo Scientific, USA
Tween 20	Vivantis Technologies, Malaysia
Yeast extract	Thermo Scientific, USA

### 3.1.2 Tools and devices

Name	Company, Country
Adhesive optical sealing film	Bioneer, South Korea
Analytical balance	Mettler Toledo, Switzerland
Autoclave	Hirayama, Japan
Autopipette	Gilson, France
Benchtop centrifuge (Hettich® Universal 320R)	Sigma-Aldrich, USA
Block heater	Wealtec, USA
Cell culture flask (25 and 75 cm <sup>3</sup> )	Corning, USA
Cell culture plate, flat bottom with lid (6-, 12-, 96-well)	Corning, USA
Cell culture plate, black, flat bottom with lid (96-well)	Corning, USA
Centrifuge tube (15 and 50 mL)	Corning, USA
Centrifugal evaporator (miVac Quattro)	Genevac, UK





CO <sub>2</sub> incubator (Forma Series II 3110)	Thermo Scientific, USA
Confocal laser scanning microscope (LSM 700)	Carl Zeiss, Germany
Cryovial tube (2 mL)	Corning, USA
Disposable serological pipettes (5, 10, 25 mL)	Corning, USA
Electrophoresis power supply	Bio-Rad, USA
Extraction thimble cellulose (Whatman <sup>TM</sup> )	GE Healthcare, USA
Filter tips (ART <sup>®</sup> 10, 100, 200, 1000 µL)	Thermo Scientific, USA
Filter paper no.1 (Whatman <sup>TM</sup> )	GE Healthcare, USA
Flow cytometer (BD FACSCalibur <sup>TM</sup> )	BD Bioscience, Germany
Fluorescence microscope (Axio Observer A1)	Carl Zeiss, Germany
Freezer (-20°C)	Sanyo Electric, Japan
Freezer (-80°C)	Lyofreeze, USA
Fused-silica capillary column (Agilent HP-5MS, 30 m × 0.25 mm, i.d., 0.25 µm film thickness)	Agilent Technologies, USA
Gas chromatograph (Agilent 6890)	Agilent Technologies, USA
Gel documentation (Gel doc) system	Syngene, UK
Gel electrophoresis apparatus	Bio-Rad, USA
Metal-cased heating mantle (LabHeat <sup>®</sup> )	SciQuip, UK
Hemocytometer	Hausser Scientific, USA





Incubator	Memmert, Germany
Incubation shaker (Minitron)	INFORS HT, Switzerland
Inverted microscope	Olympus Optical, Japan
Laboratory glass bottles (Duran <sup>®</sup> )	DWK Life Sciences, Germany
Laminar flow cabinet	Haier, China
Laminar flow cabinet	ESI Flufrance, France
Laminar flow clean bench	Esco, Singapore
Light microscope	Olympus Optical, Japan
Liquid nitrogen tank	Taylor Wharton, USA
Mass selective detector (Agilent 5973)	Agilent Technologies, USA
Micro high speed refrigerated centrifuge (VS-15000CFNII)	Vision Scientific, South Korea
Microcentrifuge tube (0.2 and 0.6 mL)	Axigen Scientific, USA
Microcentrifuge tube (1.5 mL)	Biologix Research, USA
Microplate reader (EnSpire <sup>®</sup> multimode)	Perkin-Elmer, USA
Microplate reader (Synergy <sup>™</sup> Mx)	BioTek Instruments, USA
Mini Trans-Blot <sup>®</sup> Electrophoretic Transfer cell	Bio-Rad, USA
Multichannel pipette	Gilson, France
PCR tube (0.2 mL opaque white 8-strip)	Bioneer, South Korea



pH meter	Mettler Toledo, Switzerland
Pipette Controller	Eppendorf, Germany
Pipette tips (10 µL)	Sorenson, USA
Pipette tips (200 and 1000 µL)	Corning, USA
Real-time quantitative thermal block (Exicycler™ 96)	Bioneer, South Korea
Refrigerator (4°C)	Sharp, Japan
Rotary evaporator (Laborota 4001)	Heidolph Instruments, Germany
Rotary evaporator (Rotavapor® R-210)	Buchi, Switzerland
Soxhlet extraction apparatus with Dimroth condenser	Lenz Laborglas, Germany
Stereomicroscope	Nikon Corporation, Japan
UV-Vis spectrophotometer (NanoDrop® ND-1000)	Thermo Scientific, USA
Vortex mixer (Genie 2)	Scientific Industries, USA
Vortex mixer (FINEVORTEX)	FinePCR, South Korea
Waterbath	Memmert, Germany



### 3.1.3 Plant materials

Five species of medicinal plants used in this study (Figure 14) were selected according to the following criteria: (1) found in Thailand, and (2) never been studied for the effect against glutamate-induced neurotoxicity, and/or (3) traditionally used as an ingredient in Thai traditional formula for longevity or for brain function improvement, and/or (4) available reported for anti-inflammation and antioxidants. The plant materials were collected from several sources in Thailand depending on their availability. All plants were botanically authenticated, and their voucher specimens were deposited in the herbarium of Kasin Suvatabhandhu, Department of Botany, Faculty of Science, Chulalongkorn University, Thailand.

1. *Morinda citrifolia* Linn.

Family: Rubiaceae

Common name: Yor-Ban (in Thai), Noni (in English)

Part used: Fruits

Source of collection: HRH Princess Maha Chakri Sirindhorn Herbal Garden, Rayong Province, Thailand

Herbarium voucher number: A013699(BCU)

2. *Caesalpinia mimosoides* Lamk.

Family: Fabaceae

Common name: Cha-rueat or Phak puya (in Thai), Mimosa Thorn (in English)

Part used: Young twigs and leaf

Source of collection: Market, Chiang Rai Province, Thailand

Herbarium voucher number: A014170(BCU)

3. *Murdannia loriformis* (Hassk.) Rolla Rao et Kammathy

Family: Commelinaceae

Common name: Yaa-puk-king (in Thai), Beijing Grass (in English)



Part used: Leaf

Source of collection: HRH Princess Maha Chakri Sirindhorn Herbal Garden, Rayong Province, Thailand

Herbarium voucher number: A013413(BCU)

4. *Acanthus ebracteatus* Vahl.

Family: Acanthaceae

Common name: Nguag-plaa-moa (in Thai), Sea holly (in English)

Part used: Leaf

Source of collection: HRH Princess Maha Chakri Sirindhorn Herbal Garden, Rayong Province, Thailand

Herbarium voucher number: A013422(BCU)

5. *Streblus asper* Lour.

Family: Moraceae

Common name: Khoi (in Thai), Siamese rough bush or Tooth brush tree (in English)

Part used: Leaf

Source of collection: HRH Princess Maha Chakri Sirindhorn Herbal Garden, Rayong Province, Thailand

Herbarium voucher number: A013419(BCU)







Figure 14: Five species of Thai medicinal plants used in this study.

Selected plants include (A) *Morinda citrifolia* Linn., (B) *Caesalpinia mimosoides* Lamk. [Image from Sireeruckhachati Nature Learning Park], (C) *Murdannia loriformis* (Hassk.) Rolla Rao et Kammathy, (D) *Acanthus ebracteatus* Vahl., and (E) *Streblus asper* Lour.



### 3.1.4 Cell line

The immortalized mouse hippocampal HT-22 cell line, served as an *in vitro* model for the study of glutamate-induced oxidative neurotoxicity, was a generous gift from Professor David Schubert at the Salk Institute, San Diego, CA, USA. The HT-22 cell line is originally a glutamate-sensitive subclone of the HT-4 cell line, which derived from the immortalization of primary mouse hippocampal neuronal tissues with a temperature sensitive SV40 T-antigen [365, 366]. The HT-22 cells are adherent, and their normal morphology in culture is shown in Figure 15.

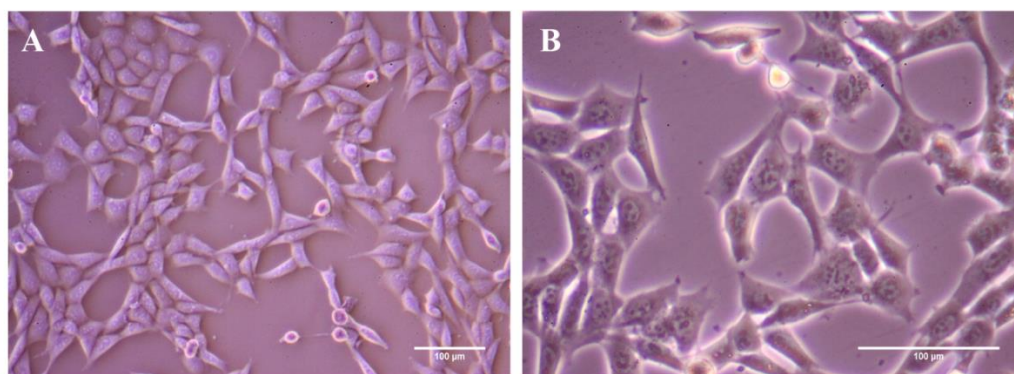


Figure 15: Normal morphology of HT-22 cells observed under phase contrast microscope at (A) 5X and (B) 10X magnification

### 3.1.5 Nematode model organism

The nematode *Caenorhabditis elegans* Bristol N2 strain was served as an *in vivo* model for the study of longevity and aging due to its rapid life cycle and ease of culture. The Bristol N2 strain is considered as a standard wild-type laboratory strain since thousands of *C. elegans* mutant strains have been derived from the Bristol N2 background [367]. This strain was originally isolated from a mushroom compost pile near Bristol, UK in 1951, and has been cultivated in the laboratory for decades [368, 369]. Figure 16 shows *C. elegans* Bristol N2 strain at different stages of development in culture.



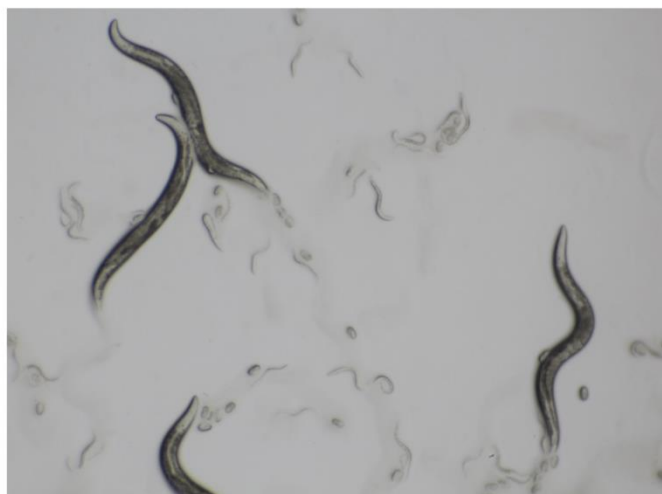


Figure 16: A population of *C. elegans* Bristol N2 strain in a mixed developmental stage on the surface of agar plate observed under a stereo-microscope at 5X magnification

### 3.1.6 Bacterial model organisms

The bacterial model organisms used for the analysis of the antibacterial activity of plant extracts in this study consisted of the following four bacterial strains: Gram-positive bacteria: *Staphylococcus aureus* ATCC 25923 and *Bacillus subtilis* SU5; Gram-negative bacteria: *Escherichia coli* MG1655 and *Pseudomonas aeruginosa* PA14.

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## 3.2 Methods

### 3.2.1 Preparation of plant extracts

After collection, the plant materials were washed, air-dried under shade, and ground finely in a mechanical grinder. The extraction was carried out by two conventional extraction techniques, maceration and Soxhlet (Figure 17), using at least two different polarities of solvents per plant. The extracts of *Morinda citrifolia* (MC) fruits and *Caesalpinia mimosoides* (CM) young twigs and leaf were prepared using Soxhlet extraction procedure. In the process of Soxhlet extraction, about 40 g of the dried powdered plant



was uniformly packed into a thimble and sequentially extracted in a Soxhlet apparatus with 400 mL of three different solvents; hexane, ethyl acetate, methanol or ethanol for at least 24 h per solvent. The extracts of *Murdannia loriformis* (ML) leaf, *Acanthus ebracteatus* (AE) leaf, and *Streblus asper* (SA) leaf were prepared using maceration. For maceration method, the coarsely powdered plant material was soaked in hexane or ethanol at a ratio 1:10 (w/v) under agitation at room temperature (RT) for 48 h. This process was performed twice, and all resulting supernatants were combined. Subsequently, the collected liquid extracts obtained after maceration and Soxhlet extractions were filtered and evaporated to dryness under vacuum. Finally, the resulting residues were prepared as a stock solution of 100 mg/mL by re-dissolved in dimethyl sulfoxide (DMSO), sterilized through a 0.2  $\mu\text{m}$  pore size syringe filter, stored at  $-20^{\circ}\text{C}$ , and protected from light until further use.



Figure 17: Plant extract preparation using (A) Soxhlet apparatus  
and by (B) maceration in a shaking incubator



### 3.2.2 Cell culture

HT-22 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS), and antibiotic solution (100 units/mL penicillin and 100 µg/mL streptomycin) in a humidified atmosphere of 5% CO<sub>2</sub> at 37°C. Upon reaching about 80% confluence, the cells were subcultured into fresh medium to maintain their exponential growth or were seeded to perform the experiments. The subculture or cell seeding procedure was carried out by washing the cells with PBS twice, detaching the cells with trypsin-EDTA solution, counting the cells with trypan blue and hemocytometer, and finally transferring the cells to grow in a new culture flask or multiwell culture plate as desired.

### 3.2.3 Determination of cytotoxicity

The cytotoxicity effects of glutamate, or plant extracts, or glutamate combined with plant extracts in HT-22 cells were assessed using two conventional colorimetric methods as follows.

#### 3.2.3.1 MTT reduction assay

The MTT reduction assay was used to evaluate cytotoxicity based on the determination of mitochondrial NAD(P)H-dependent oxidoreductase activity in metabolically active cells to reduce the yellow tetrazolium salt, 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium Bromide (MTT), into a purple formazan product. HT-22 cells were seed at a density of  $6 \times 10^3$  cells/well in 96-well culture plate and incubated overnight prior to treatment with 5 mM glutamate alone, or plant extracts alone, or 5 mM glutamate in combination with various concentrations of plant extracts for 24 h. The MTT was prepared as a stock solution of 5 mg/ml in phosphate buffered saline (PBS) at pH 7.2, sterilized through a 0.2 µm pore size syringe filter, protected from light, and stored at -20°C. At the end of the exposure period, the MTT stock solution was added into each well



at final concentration of 0.5 mg/mL and incubated for additional 4 h at 37°C in the dark. Afterwards, the culture medium and MTT solution were carefully removed from the wells, and the generated purple formazan crystals were then dissolved in DMSO. Finally, the absorbance was determined at 550 nm by using a microplate reader (BioTek Instruments) and is proportional to the number of viable cells. The percentage of cell viability was calculated by the following formula: Cell viability (%) = [Abs of treated cells/ Abs of untreated cells (control)] × 100. Results were expressed as a percentage relative to untreated cells (control).

### 3.2.3.2 LDH leakage assay

The LDH leakage assay was used to evaluate cytotoxicity based on the determination of the release of cytoplasmic lactate dehydrogenase (LDH) from damaged cells to convert the colourless tetrazolium salt, 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride (INT), into a red formazan product. LDH is a stable cytosolic enzyme present in all cell types that is rapidly released into extracellular space upon the damage of plasma membrane, and is, therefore, a good indicator of cellular toxicity. HT-22 cells were seed at a density of  $6 \times 10^3$  cells/well in 96-well culture plate and incubated overnight prior to treatment with 5 mM glutamate alone, or plant extracts alone, or 5 mM glutamate in combination with various concentrations of plant extracts for 24 h. At the end of the exposure period, the amount of LDH released into the culture medium was measured using the CytoTox 96<sup>®</sup> non-radioactive cytotoxicity assay (Promega) according to manufacturer's instructions. In brief, the culture supernatant was incubated with reconstituted substrate mix in the dark for 30 min at RT, followed by adding the stop solution before measurement. The absorbance was then recorded by a microplate reader (BioTek Instruments) at 490 nm and is proportional to the number of damaged cells. Results are expressed as a percentage of maximum LDH release obtained by complete lysis of untreated cells (control).



### 3.2.4 Flow cytometric determination of apoptotic cells

Apoptotic cell death is generally characterized based on the externalization of phosphatidylserine to the outer cell surface, which was determined using a fluorescein isothiocyanate (FITC) Annexin V apoptosis detection kit with propidium iodide (PI) (BioLegend) according to manufacturer's protocol. The kit uses Annexin V conjugated with a green fluorescent FITC to selectively label phosphatidylserine on the membrane surface of apoptotic cells in the presence of calcium ions. Simultaneously, the red fluorescent PI is used to label the cellular DNA in late apoptotic cells (as well as necrotic cells) where the integrity of plasma and nuclear membranes has been decreased (Figure 18). HT-22 cells were seed onto a 6-well culture plate at a density of  $1.5 \times 10^5$  cells/well and incubated overnight prior to treatment with 5 mM glutamate alone or 5 mM glutamate in combination with 50  $\mu\text{g/mL}$  of the plant extracts for 18 h. HT-22 cells exposed to 1 mM  $\text{H}_2\text{O}_2$  were used as a positive control. At the end of the exposure period, the harvested cells were washed twice with phosphate-buffered saline (PBS), re-suspended in the binding buffer, and stained by the solution of FITC-conjugated Annexin V and PI for 15 min in the dark. The fluorescence intensity of stained cells, at least 10,000 cells per group, was immediately analyzed by a BD FACSCalibur™ flow cytometer (BD Bioscience). Results are expressed as a percentage of Annexin V-positive/PI-negative cells (early apoptosis) plus Annexin V/PI-positive cells (late apoptosis).

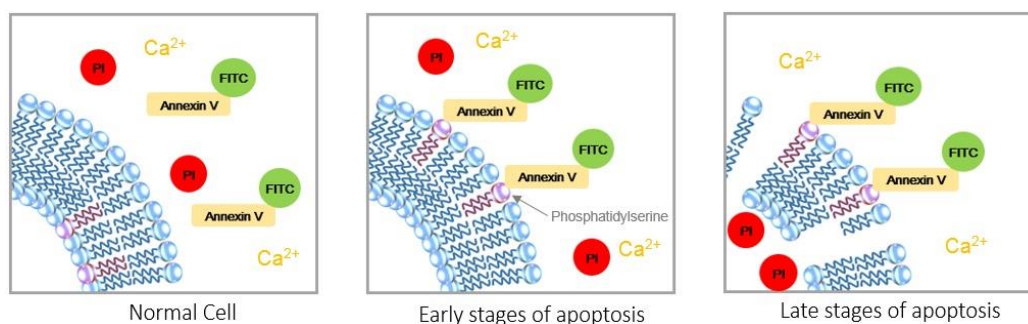


Figure 18: A schematic diagram of apoptotic detection.

During the early stage of apoptosis, the membrane lipid phosphatidylserines, which are normally deposited on the inner layer of the cell membrane, are exposed to the outer



cell surface, where they can specifically bind to the FITC-labeled Annexin V in the presence of calcium ions ( $\text{Ca}^{2+}$ ). Propidium iodide (PI) is membrane impermeant and generally excluded from viable cells. However, during the late stage of apoptosis, PI can enter the cell due to the loss of membrane integrity. (<https://www.nacalai.co.jp/global/reagent/research/annexin.html>)

### 3.2.5 Immunofluorescence microscopy

Immunofluorescence is the immunoassay technique based on the use of specific antibodies conjugated with fluorescent dyes. This technique was performed to determine the subcellular distribution of AIF in HT-22 cells following treatments. HT-22 cells were seeded in 12-well culture plate at a density of  $4 \times 10^4$  cells/well prior exposure to 5 mM glutamate alone or 5 mM glutamate in combination with 50  $\mu\text{g/mL}$  of the plant extracts for 16 h. After treatments, the cells were fixed with cold 4% (w/v) paraformaldehyde in PBS for 20 min, rehydrated in PBS for 15 min, permeabilized in 0.1% (w/v) Triton X-100 in PBS for 10 min at RT, and blocked with 5% (w/v) bovine serum albumin (BSA) in PBS for 30 min at RT. After being washed with PBS, the cells were incubated overnight at 4°C with primary antibodies against AIF (1:400; Cell Signaling Technology), followed by incubation with CF™ 555-conjugated goat anti-rabbit (1:2000; Sigma-Aldrich) for 1 h at RT. The nuclei were counterstained with 4',6-diamidino-2-phenylindole (DAPI) solution (300nM) for 10 min at RT. Following mounting with ProLong Gold antifade mountant (Thermo Scientific), stained cells were imaged using an LSM 700 confocal laser scanning microscope (Carl Zeiss).

### 3.2.6 Western Blot analysis

Western Blotting is a widely used technique to analyze specific proteins in the given sample based on detecting electrophoretically separated proteins, which were



transferred to a solid support, by using a specific primary antibody and an enzyme-conjugated secondary antibody. This analysis was used to determine the expression of targeted proteins, including apoptotic-inducing factor (AIF), nuclear factor erythroid 2-related factor 2 (Nrf2), and excitatory amino acid transporter 3 (EAAT3) in HT-22 cells following treatments. HT-22 cells were seed onto a 6-well culture plate at a density of  $2 \times 10^5$  cells/well and incubated overnight prior to treatment with 5 mM glutamate alone or 5 mM glutamate in combination with 50  $\mu\text{g/mL}$  of the plant extracts. After the indicated treatment time for each target protein, HT-22 cells were harvested, washed, and prepared for whole cell lysates to analyze EAAT3 expression as well as for cytoplasmic and nuclear fractions to analyze AIF and Nrf2 expressions. Whole-cell lysates were obtained by lysing the cells on ice for 30 min in NP-40 lysis buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1% NP-40, 1 mM PMSF, 1 mM DTT). Cytoplasmic and nuclear fractions were isolated using the NE-PER nuclear and cytoplasmic extraction reagents (Thermo Scientific) according to the manufacturer's protocol. Total protein concentrations were measured by the Bradford reagent (Bio-Rad) using bovine serum albumin (BSA) as a standard for the calibration curve. Equal amounts of proteins were denatured by boiling in 2X Laemmli loading buffer (4% (w/v) SDS; 20% (v/v) glycerol; 0.004% bromophenol blue; 0.125M Tris-Cl, pH 6.8; 10% (v/v) 2-mercaptoethanol) at 95°C for 5 min. Subsequently, the denatured proteins were separated by electrophoresis on 10% (v/v) SDS-polyacrylamide gels and electrotransferred to polyvinylidene difluoride (PVDF) membranes. The membranes were blocked for 1 h with 5% skim milk in TBS-T (Tris-buffered saline, 0.1% Tween 20) and allowed to incubate overnight at 4°C with primary antibodies specific for AIF (1:2000; Cell Signaling Technology), Nrf2 (1:2000; ; Santa Cruz Biotechnology), EAAT3 (1:8000; Abcam), Lamin B1 (1:2000; Cell Signaling Technology), or  $\beta$ -actin (1:16000; Cell Signaling Technology) and subsequently incubated for an additional 45 min at RT with horseradish peroxidase (HRP)-conjugated secondary antibodies (1:10000; Cell Signaling Technology). Specific target protein bands were visualized with enhanced chemiluminescence (ECL) detection reagent (GE Healthcare). Densitometric analysis of the bands was performed with an image analysis



system (Syngene). Results were normalized to the expression level of endogenous loading controls, Lamin B1 and  $\beta$ -actin for nuclear and cytoplasmic fractions/whole cell lysates, respectively.

### 3.2.7 Quantitative real-time reverse transcription PCR analysis

Reverse transcription PCR (RT-PCR) analysis was used to determine the expression of targeted genes at the transcriptional level. In this technique, messenger RNA is converted into complementary DNA (cDNA) which was then used in the PCR amplification. Subsequently, the amplified products were detected as the reaction progresses in "real-time" or at end-point using agarose gel electrophoresis. The mRNA expression of two major functional classes of glutamate receptors genes including metabotropic glutamate receptor type 1 (*GRM1*), NMDA-type ionotropic glutamate receptor subunits 1 (*GRIN1*), subunits 2A (*GRIN2A*), and subunits 2B (*GRIN2B*) in HT-22 cells were determined by RT-PCR. The mRNA expression level of three antioxidant-related genes including excitatory amino acid transporter 3 (*EAAT3*), NAD(P)H:quinone oxidoreductase (*NQO1*), and glutamate-cysteine ligase modifier subunit (*GCLM*) in HT-22 cells following treatments were quantified using real-time RT-PCR. The mouse  $\beta$ -actin gene (*ACTB*) was used as an internal control for normalization. HT-22 cells were seed onto a 6-well culture plate at a density of  $2 \times 10^5$  cells/well and incubated overnight prior to treatment with 5 mM glutamate alone or 5 mM glutamate in combination with 50  $\mu$ g/mL of the plant extracts for 24 h. At the end of the exposure period, total RNA from HT-22 cells in each treatment group was extracted with Trizol reagent (Invitrogen) following the manufacturer's instructions. The amount of RNA was determined by absorbance at 260 nm. 1  $\mu$ g of total RNA was used for cDNA synthesis using AccuPower RT PreMix (Bioneer) and oligo(dT)17 primer. The cDNA was used as a template for subsequent real-time PCR reactions performed on an Exicycler™ 96 real-time quantitative thermal block (Bioneer). The amplifications were done using the GreenStar™ qPCR PreMix (Bioneer) and the specific primers for *GRM1* [370], *GRIN1* [371], *GRIN2A* [371],



*GRIN2B* [371], *EAAT3*, *NOQ1*, and *GCLM* genes listed in Table 3. The thermal cycling conditions included an initial denaturation step at 95°C for 10 min, followed by 40 cycles, each consisting of 15 s of denaturation at 95°C, 15 s at 55°C (*GRM1*, *GRIN1*, *EAAT3*, *NOQ1*, *GCLM*, *ACTB*) or 60°C (*GRIN2A*, *GRIN2B*) for primer annealing, and 30 s at 72°C for chain elongation. A melting curve analysis was performed after amplification to verify the accuracy of the amplicon. The relative expression level of each target gene was normalized to  $\beta$ -actin expression and analyzed using the  $2^{-\Delta\Delta C_t}$  method.

Table 3: List of primers used in this study for RT-PCR.

Gene	Accession number	Sequence (5' → 3')	Product length (bp)
<i>GRM1</i>	NM_016976.3	Forward: TCATACGGAAAGGGGAAGTG Reverse: CAGCACAAAGATGAGGGTGA	245
<i>GRIN1</i>	NM_008169.3	Forward: CGGCTCTTGGGAAGATACAG Reverse: GAGTGAAGTGGTCGTTGG	152
<i>GRIN2A</i>	NM_008170.2	Forward: TAGACCTTAGCAGGCCCTCTC Reverse: GAGCTTTTGTCCCCAAGAGT	121
<i>GRIN2B</i>	NM_008171.3	Forward: TCATGGTGTCAGTGTTGGCCTTCT Reverse: TTCTTGTCCTCAGGCCGGAACC	121
<i>EAAT3</i>	NM_009199.2	Forward: ATGATCTCGTCCAGTTCGGC Reverse: TGACGATCTGCCCAATGCTT	202
<i>NOQ1</i>	NM_008706.5	Forward: CGACAACGGTCCTTTCCAGA Reverse: CTCCCAGACGGTTTCCAGAC	253
<i>GCLM</i>	NM_008129.4	Forward: GGAGCTTCGGGACTGTATCC Reverse: CAACTCCAAGGACGGAGCAT	236
<i>ACTB</i>	NM_007393.5	Forward: GGCTGTATCCCCTCCATCG Reverse: CCAGTTGGTAACAATGCCATGT	154



### 3.2.8 Determination of antioxidant activity

The antioxidant activities of Thai medicinal plant extracts were evaluated through *in vitro* and cell-based assays as follows.

#### 3.2.8.1 *In vitro* radical scavenging assay

##### 3.2.8.1.1 DPPH antioxidant assay

The 2,2'-diphenyl-1-picrylhydrazyl (DPPH) assay was used to evaluate *in vitro* radical scavenging activity of the sample based on its hydrogen atom- and/or electron-donating capacity to convert the stable free radical DPPH (DPPH<sup>•</sup>) into the reduced DPPH (DPPH-H), which was accompanied by a color change from purple to yellow. A working solution of stable free radical DPPH<sup>•</sup> (Sigma-Aldrich) was dissolved in ethanol to a final concentration of 0.2 mg/mL. All reaction was performed in 96-well plate. For the assay protocol, the DPPH<sup>•</sup> working solution was added to the extracted sample (1 mg/mL) at a ratio of 9:1 (v/v). The reaction mixture was incubated in the dark at RT for 15 min, and the absorbance was recorded using a microplate reader (BioTek Instruments) at 517 nm. Radical scavenging activity was expressed as the percentage inhibition of the DPPH<sup>•</sup> radicals calculated by the following equation: % Inhibition = 100 x [Abs of control - (Abs of the sample - Abs of blank) / Abs of control]. Ascorbic acid (vitamin C) at concentrations ranging from 1 to 100 µg/mL was used as a standard for calibration curve and the antioxidant capacity is expressed as vitamin C equivalent antioxidant capacity (VCEAC) in mg per g of dry weight extract.

##### 3.2.8.1.2 ABTS antioxidant assay

The 2,2'-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay was used to evaluate *in vitro* radical scavenging activity of the sample based on its hydrogen atom- and/or electron-donating capacity to neutralize the stable free radical cation ABTS (ABTS<sup>•+</sup>), which was accompanied by a color change from green to colorless. The cation radical ABTS<sup>•+</sup> working solution was generated by the oxidation of 7 mM ABTS (Sigma-Aldrich) with



2.45 mM potassium persulfate ( $K_2S_2O_8$ ) at a 1:1 (v/v) ratio. The reaction mixture was allowed to stand for 16-18 h in the dark prior to dilution with ethanol until the absorbance reached between 0.7 and 0.8 at 734 nm. All reaction was performed in 96-well plate. For the assay protocol, the  $ABTS^{*+}$  working solution was added to the extracted sample (1 mg/mL) at a ratio of 9:1 (v/v). The reaction mixture was incubated in the dark at RT for 30 min, and the absorbance was recorded using a microplate reader (BioTek Instruments) at 734 nm. Radical scavenging activity was expressed as the percentage inhibition of the  $ABTS^{*+}$  radicals calculated by the following equation: % Inhibition =  $100 \times [\text{Abs of control} - (\text{Abs of the sample} - \text{Abs of blank}) / \text{Abs of control}]$ . Ascorbic acid (vitamin C) at concentrations ranging from 1 to 100  $\mu\text{g/mL}$  was used as a standard for calibration curve and the antioxidant capacity is expressed as vitamin C equivalent antioxidant capacity (VCEAC) in mg per g of dry weight extract.

### 3.2.8.2 Cell-based antioxidant assay

The oxidative stress status can be monitored in the living cell based on the interaction of the non-fluorescent chemical compound with ROS to form the highly fluorescent product. 2',7'-Dichlorodihydrofluorescein diacetate (DCFH-DA) is the most commonly used non-fluorescent lipophilic probe to evaluate the cellular oxidative stress and overall production of ROS. This probe can passively diffuse through plasma membrane into the cytoplasm where its acetyl group is cleaved into the hydrophilic derivative, 2',7'-dichlorodihydrofluorescein (DCFH) by the action of intracellular esterases. Then, the DCFH becomes trapped inside the cell due to cellular impermeability and can be subsequently oxidized in the presence of intracellular oxidizing species including ROS (particularly  $H_2O_2$  and  $OH^\bullet$ ), yielding the highly fluorescent 2',7'-dichlorofluorescein (DCF) (Figure 19). The fluorescence signal generated is usually considered to reflect a disturbance in the redox state as well as the extent of ROS inside the cell. HT-22 cells were seed at a density of  $1 \times 10^4$  cells/well in 96-well black-wall culture plate and incubated overnight prior to



treatment with 5 mM glutamate alone, or plant extracts alone, or 5 mM glutamate in combination with various concentrations of plant extracts for 14 h. HT-22 cells exposed to 1 mM  $\text{H}_2\text{O}_2$  were served as a positive control. At the end of the exposure period, the cells were loaded with 5  $\mu\text{M}$   $\text{H}_2\text{DCFDA}$  (Molecular Probes), incubated at  $37^\circ\text{C}$  for another 30 min, and then washed three times with Hank's balanced salt solution (HBSS). Fluorescence was immediately determined using a microplate reader (Perkin-Elmer), and the photographs were obtained using an Axio Observer A1 fluorescence microscope (Carl Zeiss), with an excitation wavelength of 485 nm and an emission wavelength of 535 nm. Results are expressed as a percentage of fluorescence intensity relative to untreated cells (control).

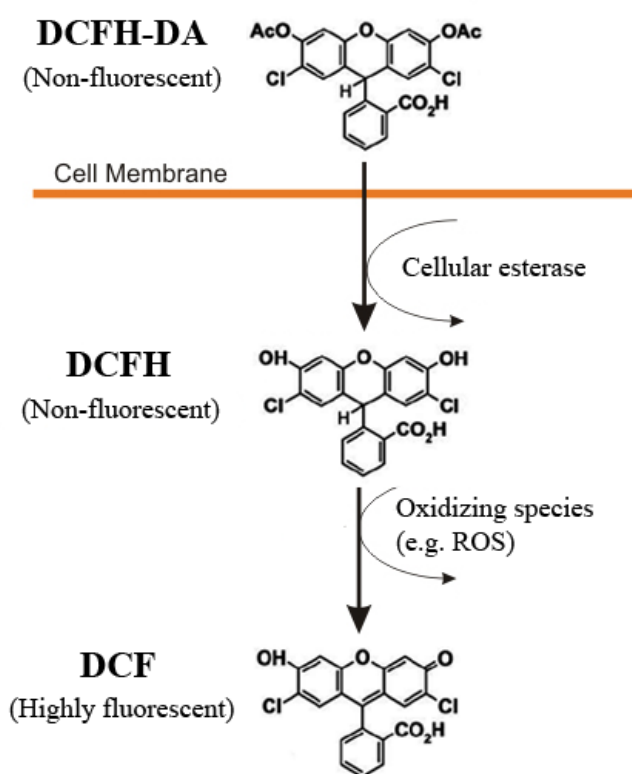


Figure 19: Mechanism of action of DCFH-DA probe inside the cell.

(adapted from [372] and ©BioTek White Papers)



### 3.2.9 Determination of phytochemical composition

#### 3.2.9.1 Determination of total flavonoid content

The total flavonoid content was determined by aluminium chloride ( $\text{AlCl}_3$ ) colorimetric method based on the formation of aluminium-flavonoid complexes under alkaline condition, which is accompanied by the appearance of yellow color. The assay was modified for a microplate format. In brief, 50  $\mu\text{L}$  of the extract (1 mg/mL) was made up to 200  $\mu\text{L}$  with 95% ethanol, and mixed well with 10  $\mu\text{L}$  of 10% (v/v)  $\text{AlCl}_3$  solution and 10  $\mu\text{L}$  of 1M sodium acetate ( $\text{NaOAc}$ ) solution. Then the mixture was allowed to stand for 40 min in the dark, and the absorbance was measured at 415 nm using a microplate reader (BioTek Instruments). Quercetin was used as a standard for the calibration curve and total flavonoid content was expressed as mg of quercetin equivalent (QE) per g of dry weight plant extract.

#### 3.2.9.2 Determination of total phenolic content

The total phenolic content was determined by the Folin-Ciocalteu method based on the reduction of the mixture of phosphomolybdic and phosphotungstic acids (Folin-Ciocalteu's phenol reagent) by the phenolic compounds under alkaline condition, which results in the formation of a blue colored chromogen constituted by the blue oxides of tungsten and molybdenum. The assay was modified for a microplate format. Briefly, 50  $\mu\text{L}$  of the extract (1 mg/mL) was mixed thoroughly with 50  $\mu\text{L}$  of a 10-fold diluted Folin-Ciocalteu's phenol reagent. After 20 min, the mixture was made alkaline by addition of 50  $\mu\text{L}$  of a 7.5% (w/v) sodium carbonate ( $\text{Na}_2\text{CO}_3$ ) solution and then kept in the dark at RT for a further 20 min. Finally, the absorbance was measured at 760 nm using a microplate reader (BioTek Instruments). Gallic acid was used as a standard for the calibration curve and total phenolic content was expressed as mg of gallic acid equivalent (GAE) per g of dry weight plant extract.



### 3.2.9.3 LC-MS analysis

Liquid chromatography-mass spectrometry (LC-MS) is a powerful analytical technique to identify the phytochemical constituents present in the plant extracts. This technique combines the physical separation capability of liquid chromatography (or high-performance liquid chromatography; HPLC) with the mass analysis capability of mass spectrometry (MS) (Figure 20). Liquid chromatography separates a mixture into individual components by the differences in their attraction to a solid stationary phase and a liquid mobile phase based on each component's properties such as polarity and solubility. Subsequently, these components flow into a mass spectrometer where they are ionized into the molecular ions ( $[M]^+$  or  $[M]^-$ ) or the pseudo-molecular ions (often as  $[M+H]^+$  or  $[M-H]^-$ ) and their mass/charge ( $m/z$ ) ratios are determined.

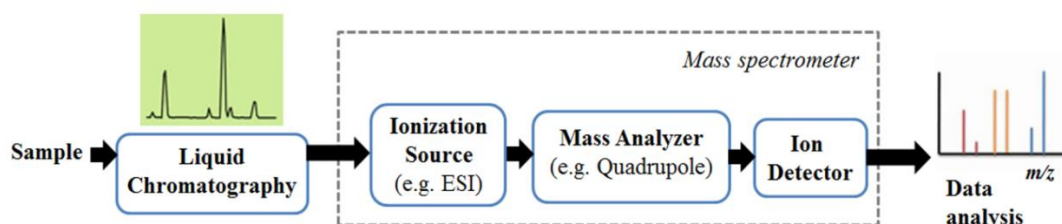


Figure 20: A schematic diagram of LC-MS system.

In the present study, the plant extracts were submitted to Institute of Systems Biology (University Kebangsaan Malaysia, Malaysia) for the screening of phytochemical constituents using LC-MS analysis. The analytical system used was a Dionex™ UltiMate 3000 UHPLC system (Thermo Fisher Scientific) coupled with a high-resolution micrOTOF-Q III (Bruker Daltonik GmbH, Bremen, Germany). The chromatographic separation was performed on an Acclaim™ Polar Advantage II C18 column (3 mm x 150 mm, 3  $\mu$ m particle size) (Thermo Fisher Scientific) with a gradient mobile phase consisting of 0.1% formic acid in water (solvent A) and 100% acetonitrile (solvent B). The elution program was as follows: 5% B (0-3min); 80% B (3-10min); 80% B (10-15min) and 5% B (15-22min). The flow rate was 400  $\mu$ L/min within 22 min total run-time and the injection volume was 1  $\mu$ L.



The MS instrument was operated in the positive electrospray ionization (ESI) mode with the parameters of the setting as follows: drying gas flow at 8 L/min, drying gas temperature at 200 °C, nebulizer pressure at 1.2 bar, capillary voltage at 4500 V, and  $m/z$  scan range of 50 to 1000. For identification of putative compounds, the observed (experimental)  $m/z$  values were compared with the METLIN and the KNApSACK databases as well as with the calculated (theoretical) mass values from previously published data available, with an accepted difference of less than 30 parts-per-million (ppm). Relative amount is expressed as the percentage of peak area relative to the total area of all peaks observed in the chromatogram.

#### 3.2.9.4 GC-MS analysis

Gas chromatography-mass spectrometry (GC-MS) is a commonly used technique for the analysis of small, volatile, semivolatile, and nonpolar compounds present in the plant extract. Similarly, as LC-MS, this technique combines the separation capability of gas chromatography with the mass analysis capability of mass spectrometry (MS) (Figure 21). Gas chromatography separates the sample components separates a mixture into individual components based on their relative boiling points (volatilities) and their interaction with the stationary phase, resulting in the different partition of each component between a solid stationary phase and a gaseous mobile phase (often called the carrier gas). Subsequently, these components flow into a mass spectrometer where they are ionized into the molecular ions ( $[M]^+$  or  $[M]^-$ ) and their mass/charge ( $m/z$ ) ratios are determined.



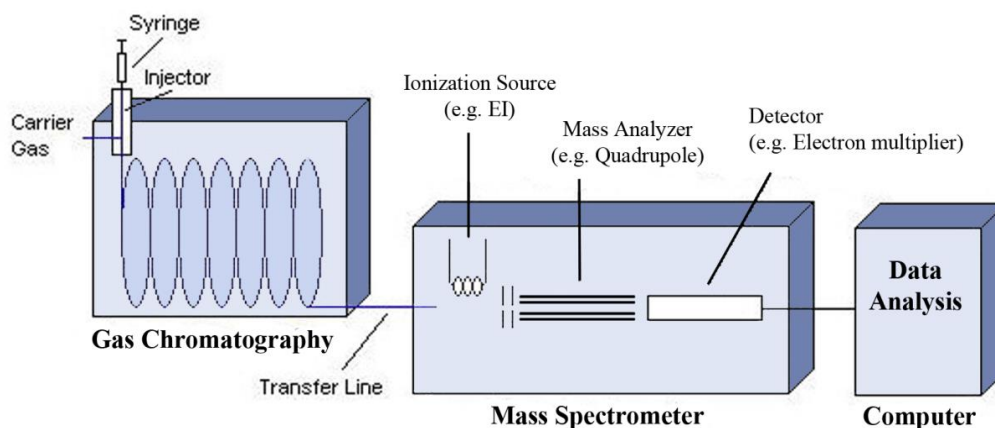


Figure 21: A schematic diagram of GC-MS system.

(modified from [373])

In the present study, analysis of volatile chemical constituents in the plant extracts was performed using Agilent 6890 gas chromatograph fitted with an Agilent HP-5MS fused-silica capillary column (5% polysilphenylene, 95% polydimethylsiloxane column, 30 m × 0.25 mm, i.d. 0.25 µm film thickness), coupled to an Agilent 5973N mass selective detector with electron-ionization (EI) source (Agilent Technologies). The samples were dissolved in ethyl acetate and injected automatically in the split mode with a split ratio of 1:25 and injection volume of 1 µL. Helium was used as the carrier gas at a constant flow rate of 1.2 mL/min. The total run time was 10.8 min. The column oven temperature was programmed initially at 50°C for 2 min. Then, it was raised to 290°C with a rate of 50°C/min and held at 290°C for 4 min. The mass spectrometer was operated with ionization energy of 70 eV, ion source temperature of 230°C, detector temperature of 150°C, and in the scan mode over the mass range of  $m/z$  40–450. The compounds were identified by matching their recorded retention times and mass spectral patterns with the NIST mass spectral library (NIST08.L).

### 3.2.10 *C. elegans* lifespan assay

Lifespan is the length of time for which individual organisms of a particular species can live. The nematode *C. elegans* is one of the principle model organisms used in the



study of lifespan because of its ease of culture, the short lifespan of three weeks, and well-characterized lifespan-regulating pathways [164, 374, 375]. The *C. elegans* Bristol N2 strain was maintained at 20°C on the surface of nematode growth medium (NGM) agar plate (50 mM NaCl, 0.25% (w/v) Peptone, 2% (w/v) agar, 1 mM MgSO<sub>4</sub>, 1 mM CaCl<sub>2</sub>, 5 µg/mL cholesterol, 25 mM KPO<sub>4</sub> buffer pH 6.0) and supplemented with *Escherichia coli* OP50 as a food source according to standard procedures [310, 376]. Before the experiment, an age-synchronized population of *C. elegans* at the first larval stage (L1) was obtained by bleaching technique that allows all the worms to be killed except for the eggs, which are protected by their shell. Gravid hermaphrodites were bleached to release their eggs by treating with a bleach solution (0.6% NaOCl (or household bleach) and 0.1 M NaOH). The remaining eggs were collected by centrifugation, washed with M9 buffer (22 mM KH<sub>2</sub>PO<sub>4</sub>, 42 mM Na<sub>2</sub>HPO<sub>4</sub>, 86 mM NaCl, 1 mM MgSO<sub>4</sub>), and grown on NGM agar plate without bacteria at 20°C overnight. To acquire worms at the fourth larval stage (L4), synchronized L1 larvae were transferred onto NGM agar plate containing *E. coli* OP50 and incubated for 40 h at 20°C. For the lifespan assay, the synchronized L1 or L4 larvae of *C. elegans* Bristol N2 strain were plated on NGM agar plates containing a lawn of *E. coli* OP50 supplemented with different concentrations of the tested extracts. The NGM plates were also contained 50 µM of 5'-fluorodeoxyuridine (FUdR) to prevent the growth of progeny and 0.1 mg/mL of ampicillin (Amp) to prevent foreign bacterial contamination. The worms were grown at 25°C and examined under a Nikon SMZ745Y stereomicroscope (Nikon Corporation) (Figure 22). Living and dead worms were counted daily starting from the first day (day 0) that *C. elegans* (at L1 or L4 stage) were transferred to experimental NGM plates until all individuals had died. The worms were scored as dead when they no longer respond to gentle stimulus with a platinum wire and showed no pharyngeal pumping movement. Worms with internally hatched progeny or extruded gonads were censored and must exclude from the experiment. The experiment was performed with at least 100 worms per group.



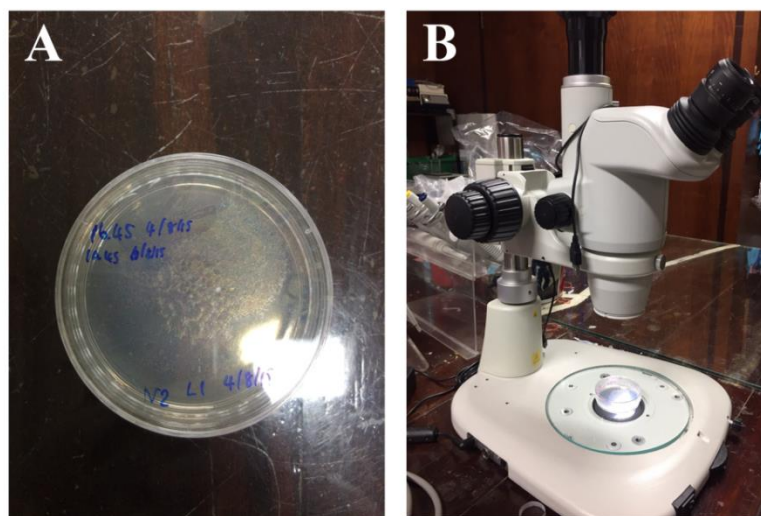


Figure 22: Image of NGM agar plate (A) and stereomicroscope (B).

### 3.2.11 Preparation of acid-base fractions

Acid-base extraction is a liquid-liquid extraction technique commonly used to separate organic compounds from a crude mixture of natural product extracts based on the difference in their acid-base properties into the acidic, basic, and neutral (non-acidic and non-basic) components. For efficient acid-base extraction, crude ethanol extract of *Streblus asper* (SA) leaf (29.44 g) was firstly dissolved in EtOAc (700 mL) and partitioned with distilled water (700 mL). The resulting organic solution was concentrated, re-dissolved in dioxane (200 mL) and slowly added dropwise of distilled water (80 mL) while stirring continuously to induce the precipitation of chlorophyll [377]. Then, the precipitates were removed by three rounds of centrifugation (4,400 rpm for 20 min) with each round being followed by filtration through filter papers. The chlorophyll-removed extracts were combined, and solvents were removed to give a thick paste. The paste was used for fractionation into neutral, acidic, and basic fractions based on the method of alkaloid extraction previously used by Mungkornasawakul et al. [378], with some modifications. The procedure of acid-base extraction was summarized in Figure 23. The crude paste was dissolved in EtOAc (150 mL) and extracted with 5% HCl solution (700 mL). The EtOAc layer



(Organic phase-1) was separated from the 5% HCl solution layer (Aqueous phase-1) and set aside for basic extraction. The 5% HCl solution was made basic (pH 10) with 10M NaOH solution, and the resulting solution was further extracted with EtOAc (200 mL x 2). The EtOAc extracts were washed with saturated NaCl solution and dried over anhydrous potassium carbonate ( $K_2CO_3$ ). The solvent was removed to afford the basic fraction.

Simultaneously, the EtOAc layer (Organic phase-1) was extracted with an equal volume of 0.5M NaOH solution. The top organic layer (Organic phase-2) was separated from the aqueous NaOH layer (Aqueous phase-2), then washed with a saturated solution NaCl and dried over anhydrous sodium sulfate ( $Na_2SO_4$ ) and the solvent was removed under reduced pressure to afford the neutral fraction. Finally, the remaining the aqueous NaOH layer (Aqueous phase-2) was then acidified to pH 1 with 5% HCl solution and extracted with EtOAc (200 mL x 2). The combined EtOAc extracts were washed with a saturated solution NaCl and dried over anhydrous  $Na_2SO_4$ , and the solvent was removed under reduced pressure to afford the acidic fraction. Parts of three fractions obtained after acid-base extraction were dissolved in DMSO, filtered through a 0.2  $\mu$ m filter, and stored at -20°C as a stock solution for evaluating biological activities.



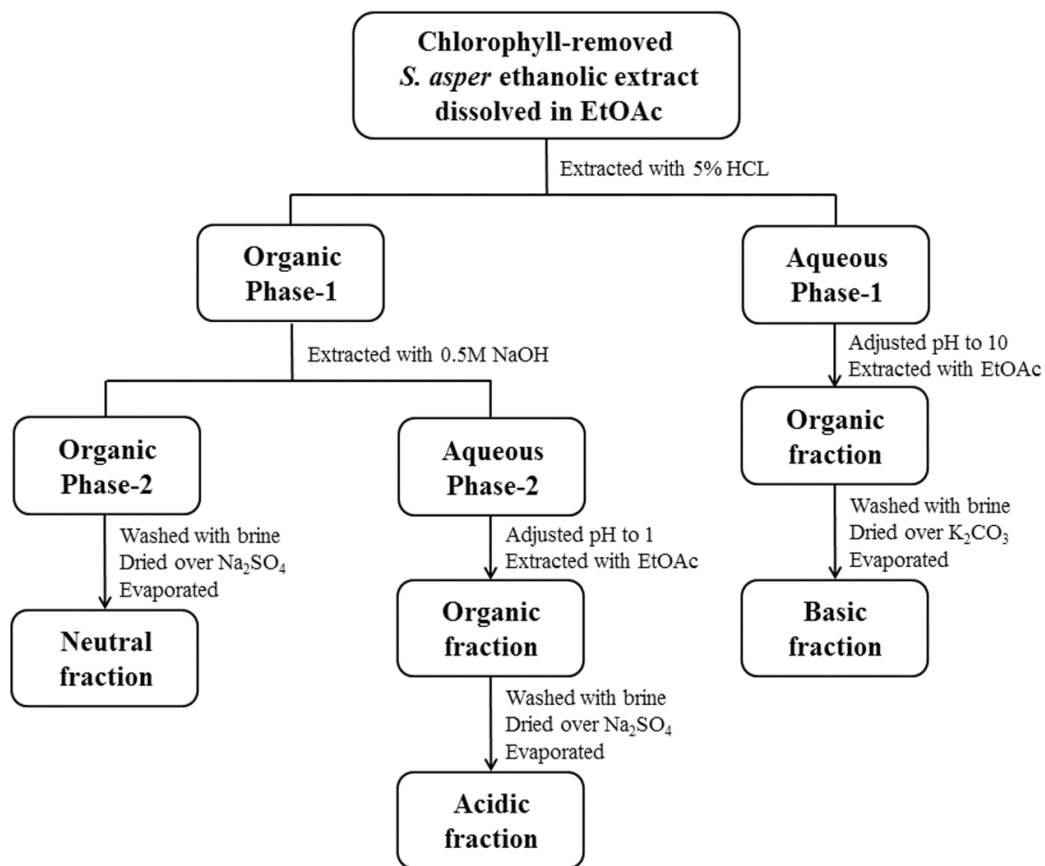


Figure 23: Acid-Base extraction of *S. asper* ethanol extract.

### 3.2.12 Determination of antibacterial activity

The antibacterial activity was determined by the broth microdilution method according to CLSI guidelines for antimicrobial susceptibility testing. The test medium was cation-adjusted Mueller-Hinton broth (CAMHB). Crude ethanol extract of *Streblus asper* (SA) leaf and its three acid-base fractions were tested against *Staphylococcus aureus* ATCC25923, *Bacillus subtilis* SU5, *Escherichia coli* MG1655, and *Pseudomonas aeruginosa* PA14. Serially diluted extract concentrations were tested using a 96-well plate, which was inoculated with a bacterium cell concentration of approximately  $5 \times 10^5$  CFU. After 20 h of incubation at 37 °C statically, the minimum inhibitory concentration (MIC) was defined as the lowest concentration of compound that showed 95% cell growth inhibition,



determined by measuring absorbance at 595 nm. The experiment was performed in biological triplicates.

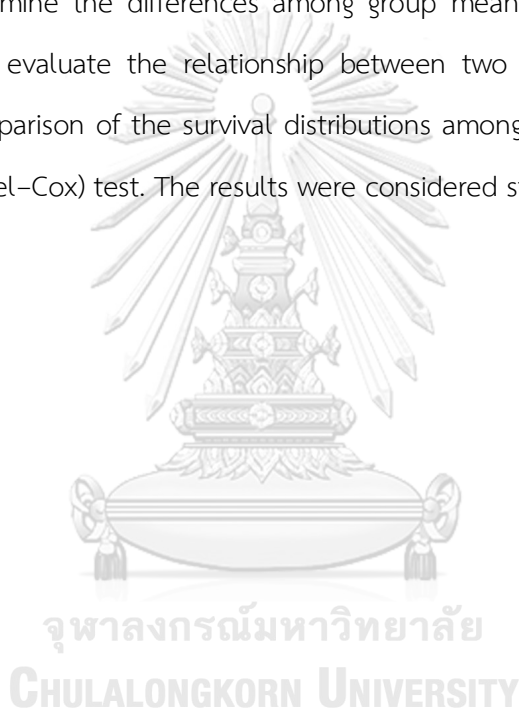
### 3.2.13 Determination of anti-acetylcholinesterase activity

The acetylcholinesterase (AChE) inhibitory activity was screened by using thin-layer chromatography (TLC)-direct bioautographic assay adapted from Marston's method [379]. This assay is based on the activity of AChE in converting the substrate 1-naphthyl acetate to 1-naphthol, which in turn reacts with the chromogenic agent Fast Blue B salt to produce a purple-coloured diazonium dye. The working solution of AChE was prepared by dissolving 1.21 mg of lyophilized powder of AChE from electric eel in 135 mL of 0.05M Tris-HCl buffer at pH 7.8 with 150 mg of bovine serum albumin. The silica gel F<sub>254</sub> TLC plates (Merck) were prewashed with acetone and thoroughly dried before use. The 1 mg of each sample was dissolved in ethyl acetate (1000 ppm), 6.7  $\mu$ L (6.7  $\mu$ g) of the solution was spotted on a pre-washed TLC plate and developed with the solvent system of hexane: ethyl acetate (7:3, v/v). Then 3.3  $\mu$ L (0.3  $\mu$ g) of galantamine solution (0.1 mg/mL, 100 ppm) was also applied on the plate as a positive control. After completely air-dried, the TLC plates were then saturated by spraying with the enzyme stock solution (6.71 U/mL) and incubated in a humidified chamber for 20 min at 37°C. Afterwards, the enzyme activity was detected by spraying with the mixture solution containing a part of 13.8 mM of 1-naphthyl acetate in ethanol plus four parts of 5.27 mM of Fast Blue B salt in distilled water, onto the moist TLC plates. The presence of potential compounds with anti-AChE activity was determined as the appearance of white (clear) spot on the purple-coloured background.



### 3.2.14 Statistical analysis

All experiments were performed in at least triplicate and the data are represented as means  $\pm$  standard deviation (SD) in figures or tables. All statistical analyses were conducted using SPSS software version 17.0 (SPSS Inc., Chicago, IL, USA) or GraphPad Prism software version 5.0 (GraphPad Software Inc., San Diego, CA, USA). Comparison between two groups was analyzed by a two-tailed unpaired Student's t-test. One-way analysis of variance (ANOVA), followed by the post hoc Tukey HSD multiple comparing tests was employed to determine the differences among group means. Pearson's correlation test was conducted to evaluate the relationship between two antioxidant assays. For the lifespan assay, comparison of the survival distributions among different groups was made by a log-rank (Mantel–Cox) test. The results were considered statistically significant when  $P < 0.05$ .





## CHAPTER IV

### RESULTS

#### 4.1 The study of neuroprotective properties of Thai medicinal plants against glutamate-induced toxicity and the underlying protective mechanisms

##### 4.1.1 Extraction yield of five Thai medicinal plants

Extraction yield is a measure of the solvent efficiency to extract specific components from the original material. It was defined as the amount of extract recovered in mass compared with the initial amount of dry plant material used in the extraction. In the present study, five Thai medicinal plants were extracted with at least two of four different polarity solvents (hexane, ethyl acetate, ethanol, and methanol) using Soxhlet or maceration extraction methods. The percentage yield of crude plant extract in each solvent was shown in Table 4, ranging from 1.48% to 37.07% (w/w). The highest and the lowest extraction yields were obtained from methanol extract of *Morinda citrifolia* (MC) fruits and hexane extract of *Murdannia loriformis* (ML) leaf, respectively.

Table 4: Extraction yield of five plants extracted in different polarity solvents.

Plant	Part used	Method	Extraction yield (% w/w)			
			Hexane (HEX)	Ethyl acetate (EtOAc)	Ethanol (EtOH)	Methanol (MET)
<i>Morinda citrifolia</i> (MC)	Fruits	Soxhlet	3.20	2.45	-	37.07
<i>Caesalpinia mimosoides</i> (CM)	Twigs/Leaf	Soxhlet	4.02	6.29	-	29.82
<i>Murdannia loriformis</i> (ML)	Leaf	Maceration	1.48	-	3.93	-
<i>Acanthus ebracteatus</i> (AE)	Leaf	Maceration	4.37	-	7.98	-
<i>Streblus asper</i> (SA)	Leaf	Maceration	3.52	-	4.03	-



#### 4.1.2 Investigation for the protective effect of Thai medicinal plant extracts against glutamate-induced cytotoxicity

##### 4.1.2.1 Expression of glutamate receptors in HT-22 cells

Before the start of experiments, the HT-22 cell line was validated whether it could be a suitable model for the study of non-receptor dependent glutamate-induced cytotoxicity. The expressions of two major functional classes of glutamate receptors, ionotropic (iGluR) and metabotropic (mGluR), in the HT-22 cells were determined at transcriptional level using reverse transcription PCR (RT-PCR). In comparison to the mouse neuroblastoma Neuro-2A (N2a) cell line which was reported of both mGluR and NMDA-type iGluR expressions [371, 380], the HT-22 cell line showed no detectable expression of mGluR type 1 (*GRM1*) and NMDA-type iGluR subunits 1 (*GRIN1*) genes, but still had the expressions of NMDA-type iGluR subunits 2A (*GRIN2A*) and subunits 2B (*GRIN2B*) genes (Figure 24). Thus, the HT-22 cell line was an appropriate cell model for the assessment of glutamate-induced cytotoxicity via non-receptor dependent pathway in the present study.

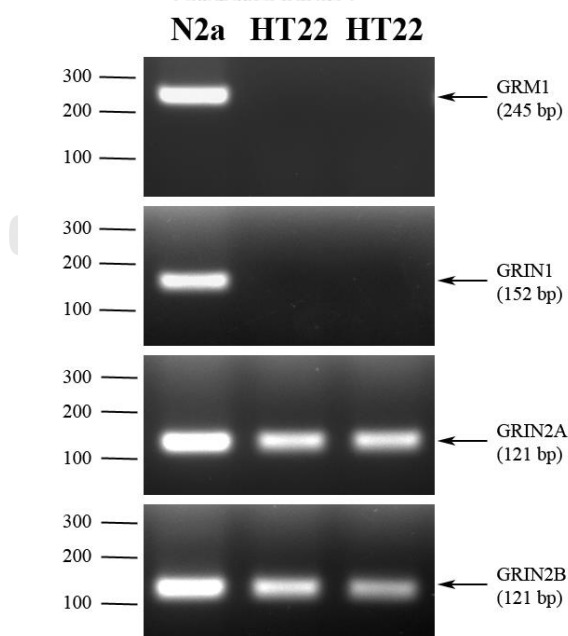


Figure 24: Expression of metabotropic and NMDA-type ionotropic glutamate receptors at the transcriptional level in N2a and HT-22 cell line determined by RT-PCR.



#### 4.1.2.2 Toxicity effect of glutamate on HT-22 cells

To obtain the optimal concentration of glutamate used in this study, the cytotoxic effect of glutamate was evaluated at its different concentrations by assessing the cell viability using MTT reduction assay. Treatment of HT-22 cells with various glutamate concentrations ranging from 0.625 to 40 mM resulted in decreased cell viability in a dose-dependent manner, which the concentration of 5 mM lead to about 50% reduction of cell viability (Figure 25A). In addition, the half-maximal Inhibitory concentration ( $IC_{50}$ ) value for glutamate is about 4.7 mM calculated by fitting the data to a sigmoidal dose-response curve using the GraphPad Prism 5 (GraphPad Software Inc., San Diego, CA, USA) (Figure 25B). Thus, the concentration of glutamate at 5 mM that induced a reduction in HT-22 cell viability of approximately 50% was chosen for subsequent experiments.

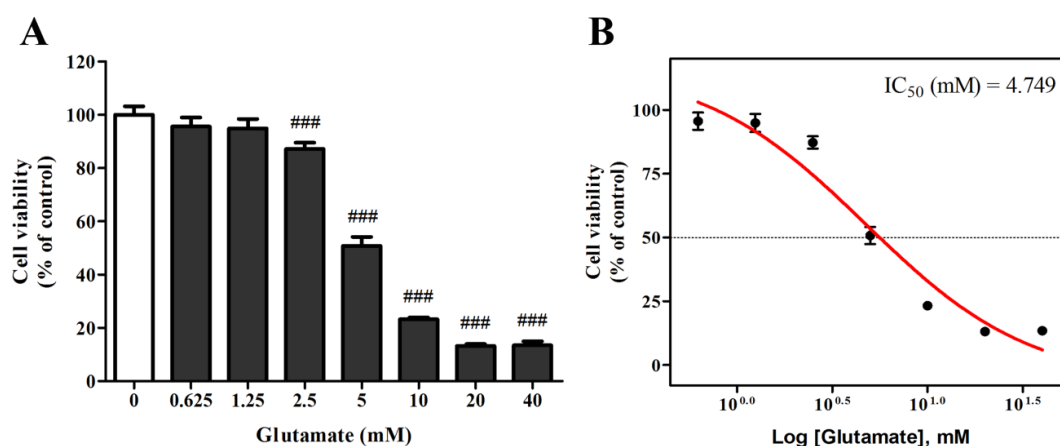


Figure 25: Effect of glutamate on the viability of HT-22 cells determined by MTT reduction assay.

HT-22 cells were exposed to a varied concentration of glutamate for 24 h. (A) Relative MTT viability and (B) the sigmoidal dose-response curve of glutamate treatment in HT-22 cells. Data are expressed as the means  $\pm$  SD of three independent experiments. <sup>###</sup> $P < 0.001$  vs. untreated cells (control).



#### 4.1.2.3 Toxicity effects of Thai medicinal plant extracts on HT-22 cells

To examine the cytotoxic effects of Thai medicinal plant extracts used in this study, the HT-22 cell viability was assessed at varying concentrations of Thai medicinal plant extracts using MTT reduction assay. Treatment of HT-22 cells with most Thai medicinal plant extracts used in this study showed the cell viability more than 80%, except for methanol extract of *Caesalpinia mimosoides* (CM). The hexane, ethyl acetate, and methanol extracts of *Morinda citrifolia* (MC) fruits at the concentrations ranging from 3.125 to 50 µg/mL did not cause any significant change in cell viability (Figure 26). The different concentrations (3.125 to 50 µg/mL) of hexane and ethyl acetate extracts of *Caesalpinia mimosoides* (CM) twigs and leaf also caused no toxic effect, however its methanol extract significantly decreased the HT-22 cell viability at the concentration of 25 and 50 µg/mL compared to untreated cells (control) (Figure 27). The hexane and ethanol extracts of *Murdannia loriformis* (ML) leaf (Figure 28), *Acanthus ebracteatus* (AE) leaf (Figure 29), and *Streblus asper* (SA) leaf (Figure 30) at the concentrations ranging from 3.125 to 50 µg/mL did not cause any noticeable toxic effect on HT-22 cells.





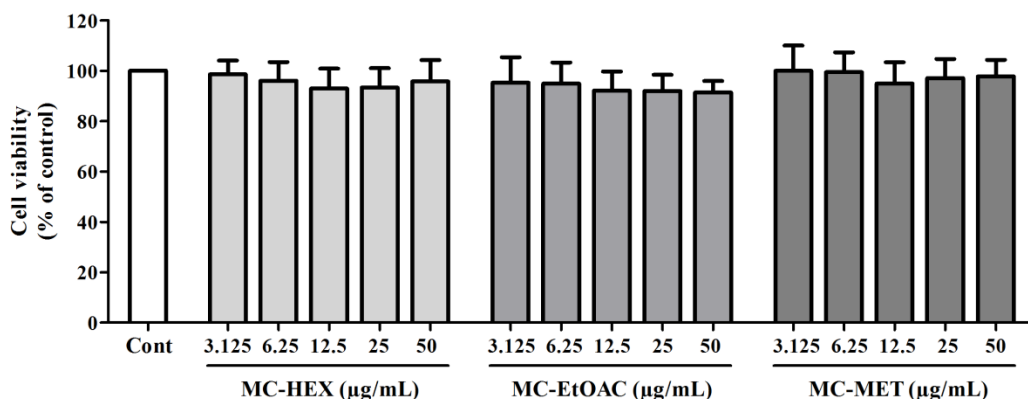


Figure 26: Effect of *Morinda citrifolia* (MC) fruits extracts

on the viability of HT-22 cells determined by MTT reduction assay.

HT-22 cells were exposed to a varying concentration of three different (hexane, ethyl acetate, and methanol) MC fruit extracts for 24 h. Data are expressed as the means  $\pm$  SD of at least three independent experiments.

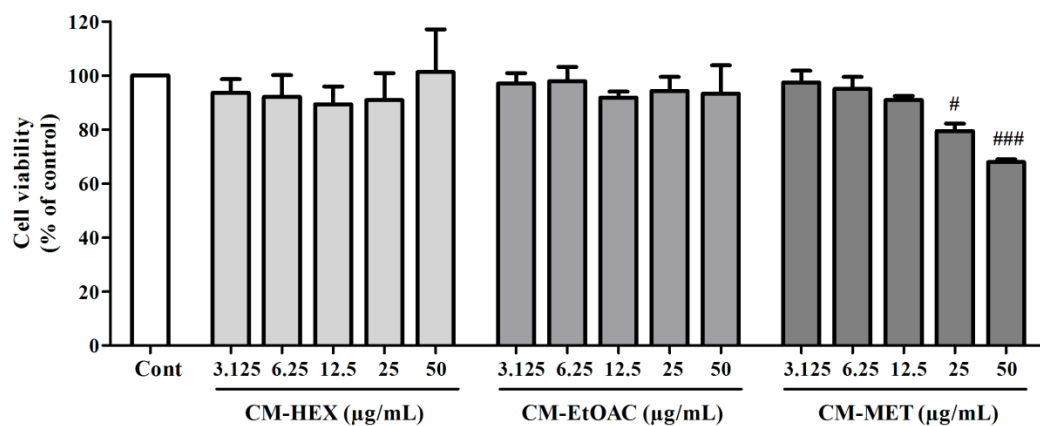


Figure 27: Effect of *Caesalpinia mimosoides* (CM) twigs and leaf extracts

on the viability of HT-22 cells determined by MTT reduction assay.

HT-22 cells were exposed to a varying concentration of three different (hexane, ethyl acetate, and methanol) CM twig and leaf extracts for 24 h. Data are expressed as the means  $\pm$  SD of at least three independent experiments. <sup>#</sup> $P < 0.05$ , <sup>###</sup> $P < 0.001$  vs. untreated cells (control).



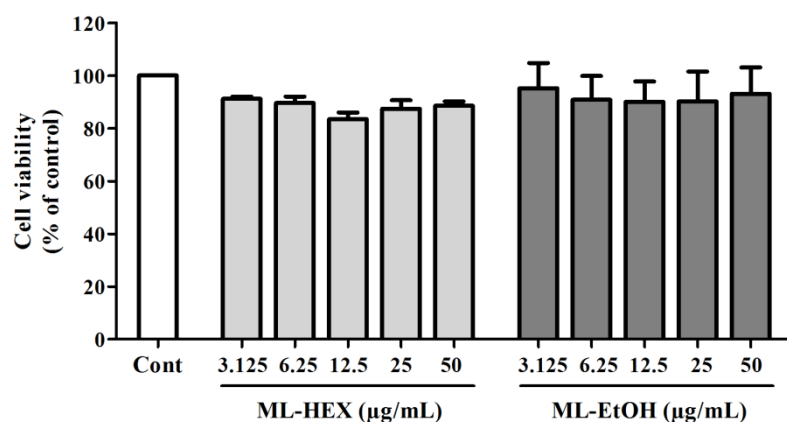


Figure 28: Effect of *Murdannia loriformis* (ML) leaf extracts on the viability of HT-22 cells determined by MTT reduction assay.

HT-22 cells were exposed to a varying concentration of two different (hexane and ethanol) ML leaf extracts for 24 h. Data are expressed as the means  $\pm$  SD of at least three independent experiments.

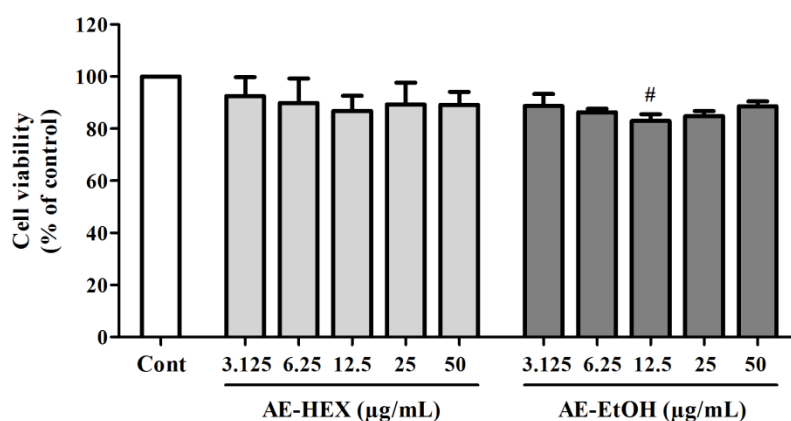


Figure 29: Effect of *Acanthus ebracteatus* (AE) leaf extracts on the viability of HT-22 cells determined by MTT reduction assay.

HT-22 cells were exposed to a varying concentration of two different (hexane and ethanol) AE leaf extracts for 24 h. Data are expressed as the means  $\pm$  SD of at least three independent experiments. <sup>#</sup> $P < 0.05$  vs. untreated cells (control).



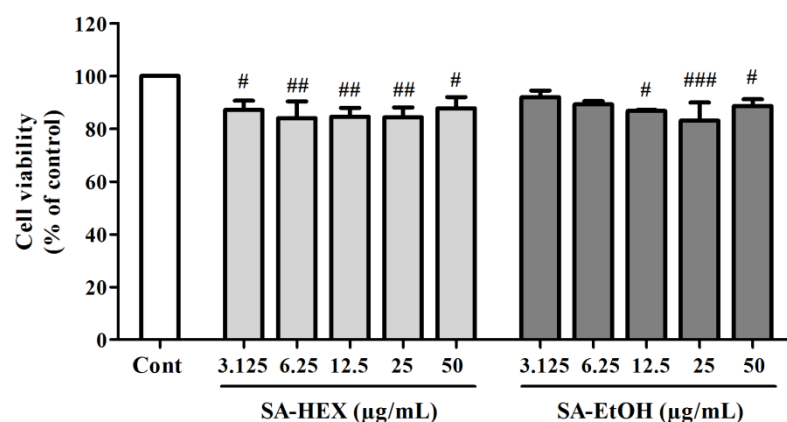


Figure 30: Effect of *Streblus asper* (SA) leaf extracts

on the viability of HT-22 cells determined by MTT reduction assay.

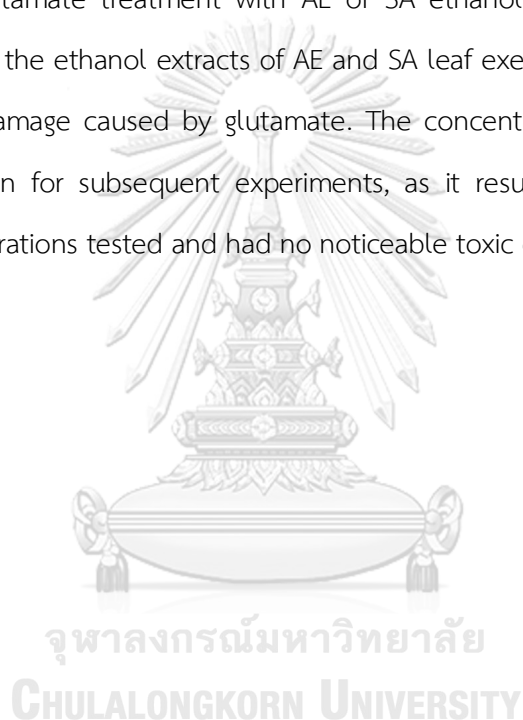
HT-22 cells were exposed to a varying concentration of two different (hexane and ethanol) SA leaf extracts for 24 h. Data are expressed as the means  $\pm$  SD of at least three independent experiments. #  $P < 0.05$ , ##  $P < 0.01$ , ###  $P < 0.001$  vs. untreated cells (control).

#### 4.1.2.4 Effects of Thai medicinal plant extracts against glutamate-induced cytotoxicity in HT-22 cells

To examine the neuroprotective effects of Thai medicinal plant extracts against glutamate-induced cytotoxicity, the viability of HT-22 cells exposed to 5 mM glutamate in the presence or absence of various extracts concentrations was assessed using MTT reduction assay and LDH leakage assay. Treatment of HT-22 cells with glutamate alone at 5 mM caused a reduction in MTT viability to about 50% of control along with an increase in LDH release to about 60% of maximum release. However, this toxic effect of glutamate could be rescued by some of Thai medicinal plant extracts used in this study, which provide different degrees of protection (Figure 31 to 35). Amongst twelve extracts of five plants, the ethanol extracts of *Acanthus ebracteatus* (AE) leaf (Figure 34), and *Streblus asper* (SA) leaf (Figure 35) had much more profound protective effects against glutamate-induced cytotoxicity. In the presence of AE or SA ethanol extracts, the glutamate-induced



cytotoxicity was significantly concentration-dependent reduced, as determined by MTT reduction (Figure 34A and 35A) and LDH leakage (Figure 34B and 35B) assays. Moreover, both AE and SA leaf ethanol extracts at the highest tested concentration of 50  $\mu\text{g/mL}$  were able to improve cell viability and restore LDH leakage to the control level. Morphological examination under a microscope also showed that glutamate-treated HT-22 cells had visible changes in their morphology including soma shrinkage to a more round shape and loss of neurites. However, these cells could be restored to normal morphology upon the combination of glutamate treatment with AE or SA ethanol extracts (Figure 36). These results suggest that the ethanol extracts of AE and SA leaf exert a potent protective effect against neuronal damage caused by glutamate. The concentration of 50  $\mu\text{g/mL}$  of both extracts was chosen for subsequent experiments, as it resulted in maximal protection among the concentrations tested and had no noticeable toxic effect on HT-22 cells.





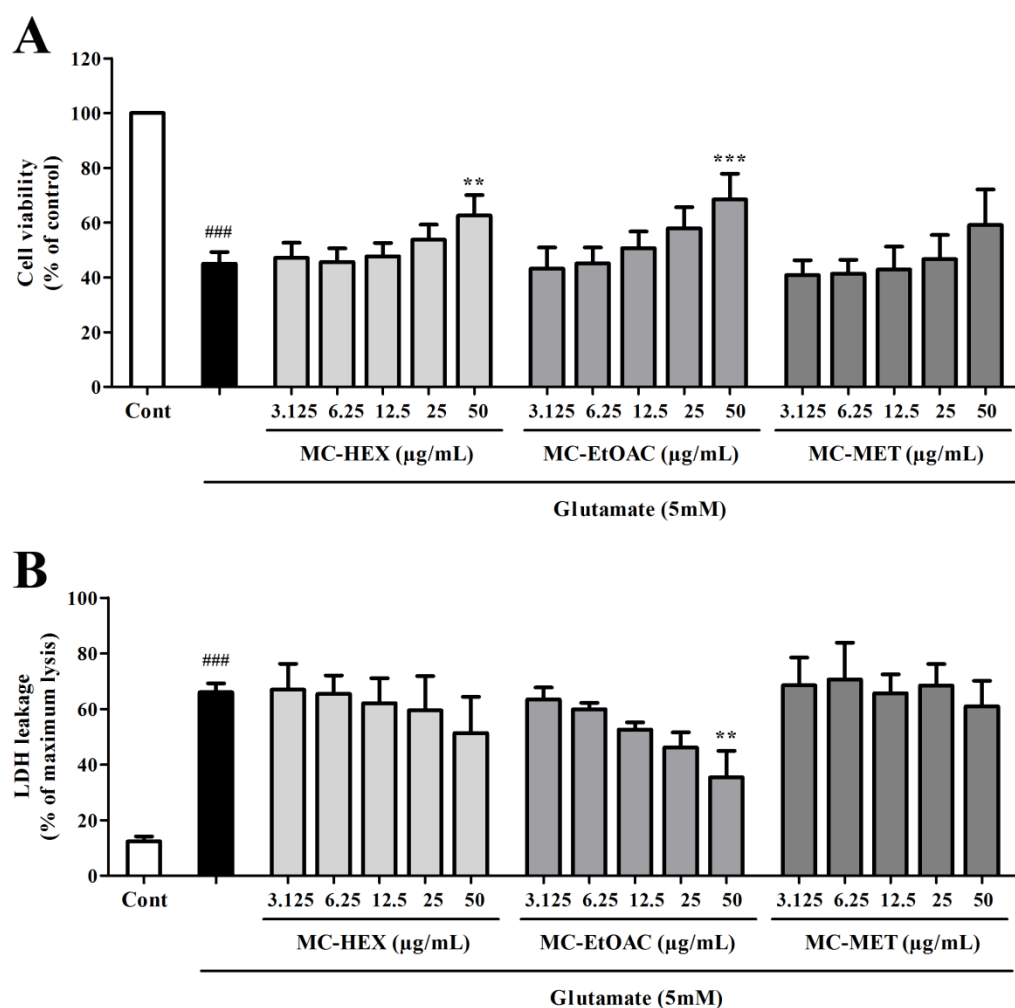


Figure 31: Protective effect of *Morinda citrifolia* (MC) fruits extracts against glutamate-induced cytotoxicity in HT-22 cells determined by (A) MTT reduction assay and (B) LDH leakage assay.

HT-22 cells were exposed to 5 mM glutamate alone or 5 mM glutamate combined with a varying concentration of three different (hexane, ethyl acetate, and methanol) MC fruits extracts for 24 h. Data are expressed as the means  $\pm$  SD of at least three independent experiments. <sup>###</sup> $P < 0.001$  vs. untreated cells (control); <sup>\*\*</sup> $P < 0.01$  vs. glutamate treatment alone.



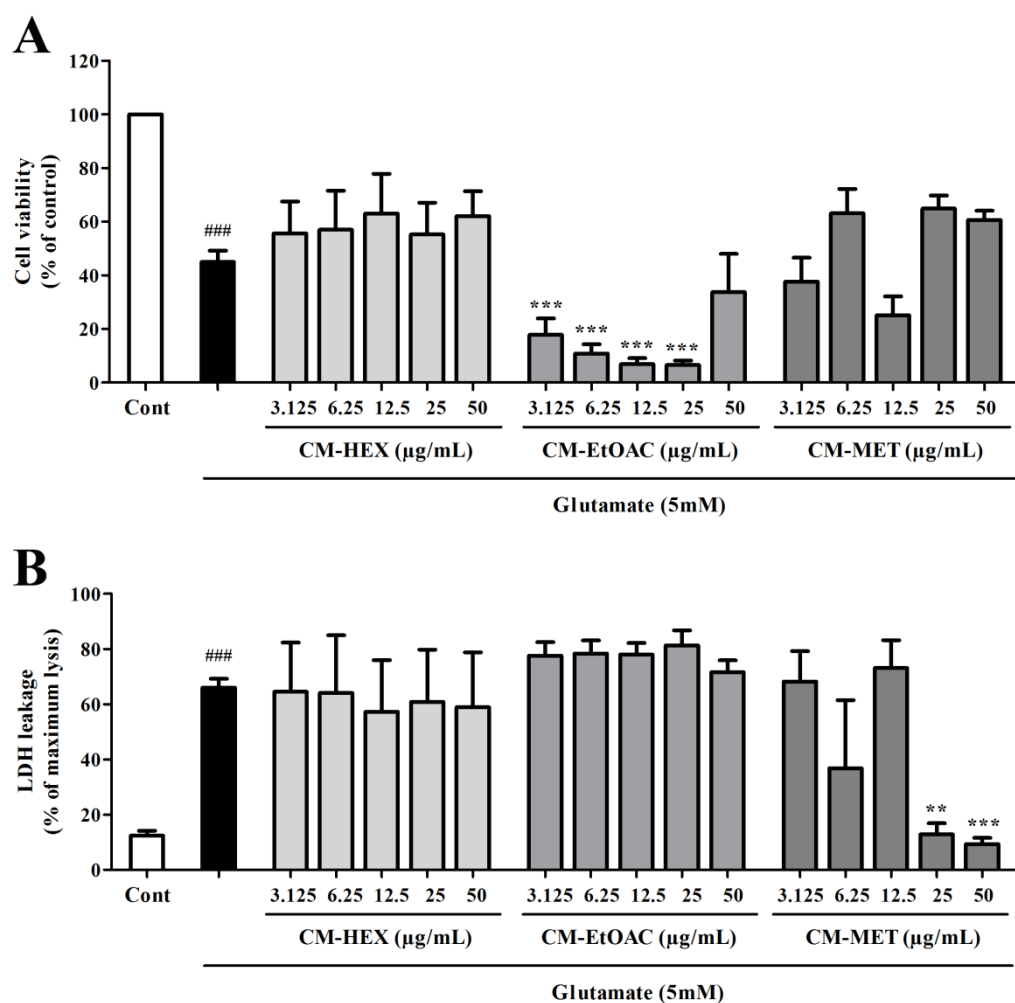


Figure 32: Protective effect of *Caesalpinia mimosoides* (CM) twigs and leaf extracts against glutamate-induced cytotoxicity in HT-22 cells determined by (A) MTT reduction assay and (B) LDH leakage assay.

HT-22 cells were exposed to 5 mM glutamate alone or 5 mM glutamate combined with a varying concentration of three different (hexane, ethyl acetate, and methanol) CM twigs and leaf extracts for 24 h. Data are expressed as the means  $\pm$  SD of at least three independent experiments.  $^{###}P < 0.001$  vs. untreated cells (control);  $^{**}P < 0.01$ ,  $^{***}P < 0.001$  vs. glutamate treatment alone.



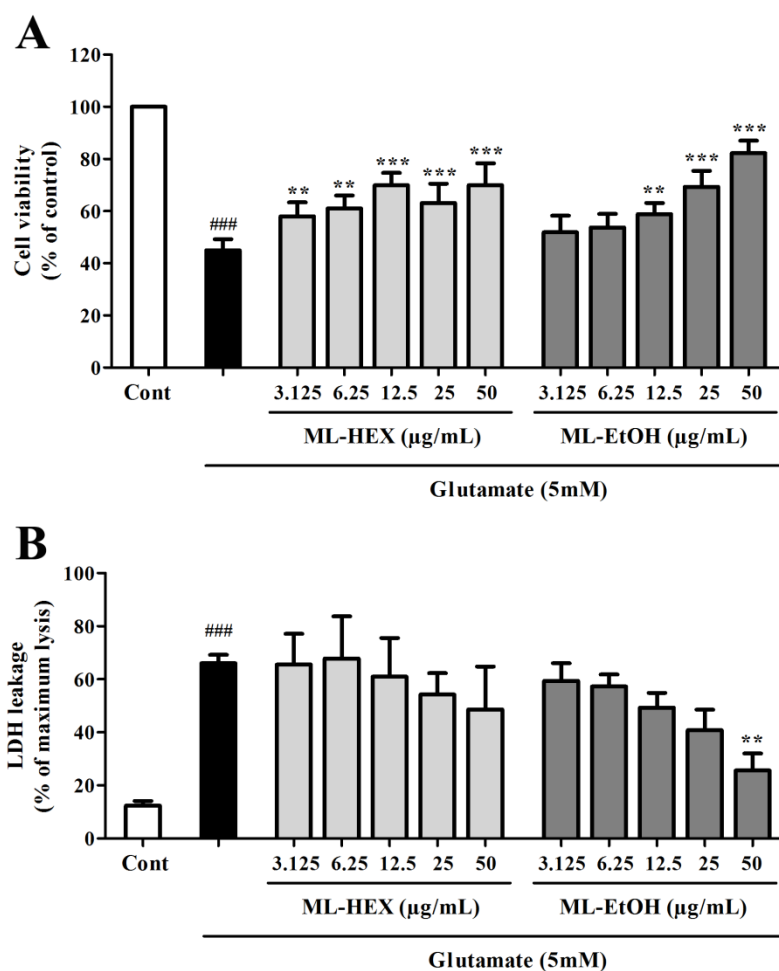


Figure 33: Protective effect of *Murdannia loriformis* (ML) leaf extracts against glutamate-induced cytotoxicity in HT-22 cells determined by (A) MTT reduction assay and (B) LDH leakage assay.

HT-22 cells were exposed to 5 mM glutamate alone or 5 mM glutamate combined with a varying concentration of two different (hexane and ethanol) ML leaf extracts for 24 h. Data are expressed as the means  $\pm$  SD of at least three independent experiments. ### $P < 0.001$  vs. untreated cells (control); \*\* $P < 0.01$ , \*\*\* $P < 0.001$  vs. glutamate treatment alone.



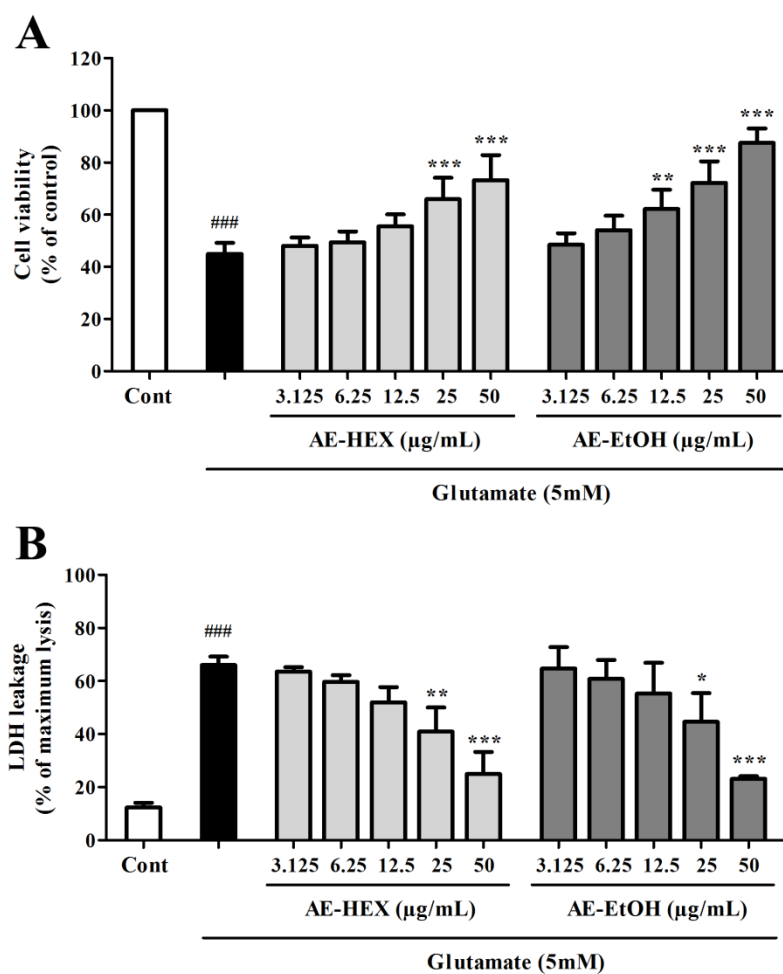


Figure 34: Protective effect of *Acanthus ebracteatus* (AE) leaf extracts against glutamate-induced cytotoxicity in HT-22 cells determined by (A) MTT reduction assay and (B) LDH leakage assay.

HT-22 cells were exposed to 5 mM glutamate alone or 5 mM glutamate combined with a varying concentration of two different (hexane and ethanol) AE leaf extracts for 24 h. Data are expressed as the means  $\pm$  SD of at least three independent experiments.  $^{###}P < 0.001$  vs. untreated cells (control);  $^{*}P < 0.05$ ,  $^{**}P < 0.01$ ,  $^{***}P < 0.001$  vs. glutamate treatment alone.



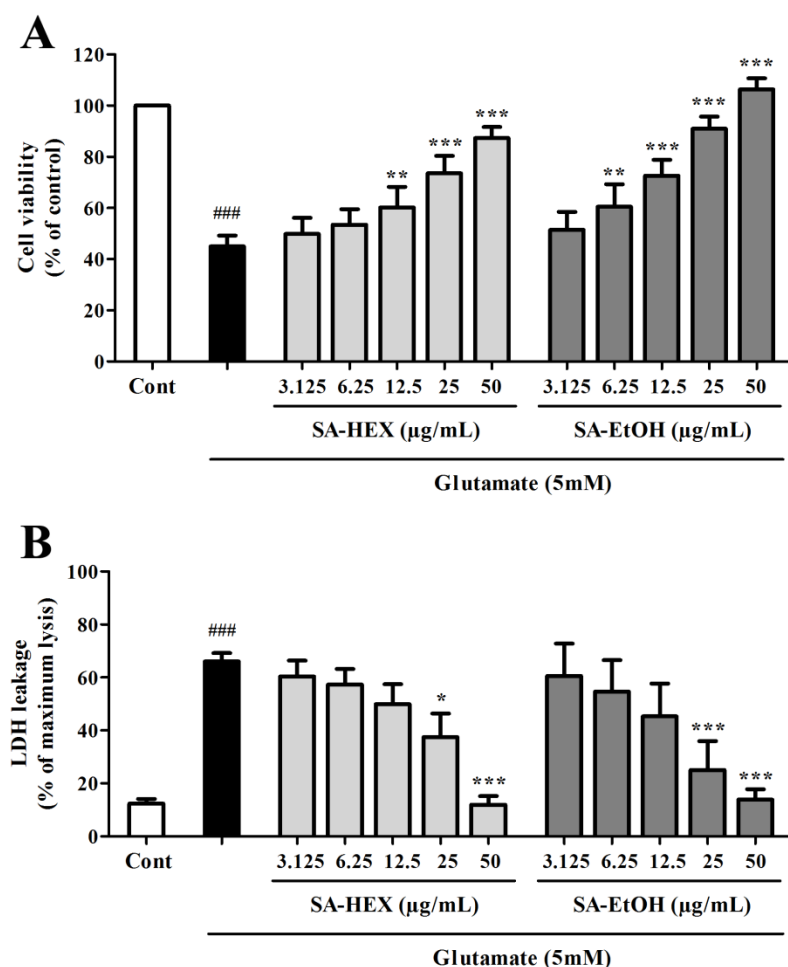


Figure 35: Protective effect of *Streblus asper* (SA) leaf extracts against glutamate-induced cytotoxicity in HT-22 cells determined by (A) MTT reduction assay and (B) LDH leakage assay.

HT-22 cells were exposed to 5 mM glutamate alone or 5 mM glutamate combined with a varying concentration of two different (hexane and ethanol) SA leaf extracts for 24 h. Data are expressed as the means  $\pm$  SD of at least three independent experiments. <sup>###</sup> $P < 0.001$  vs. untreated cells (control);  $^*P < 0.05$ ,  $^{**}P < 0.01$ ,  $^{***}P < 0.001$  vs. glutamate treatment alone.



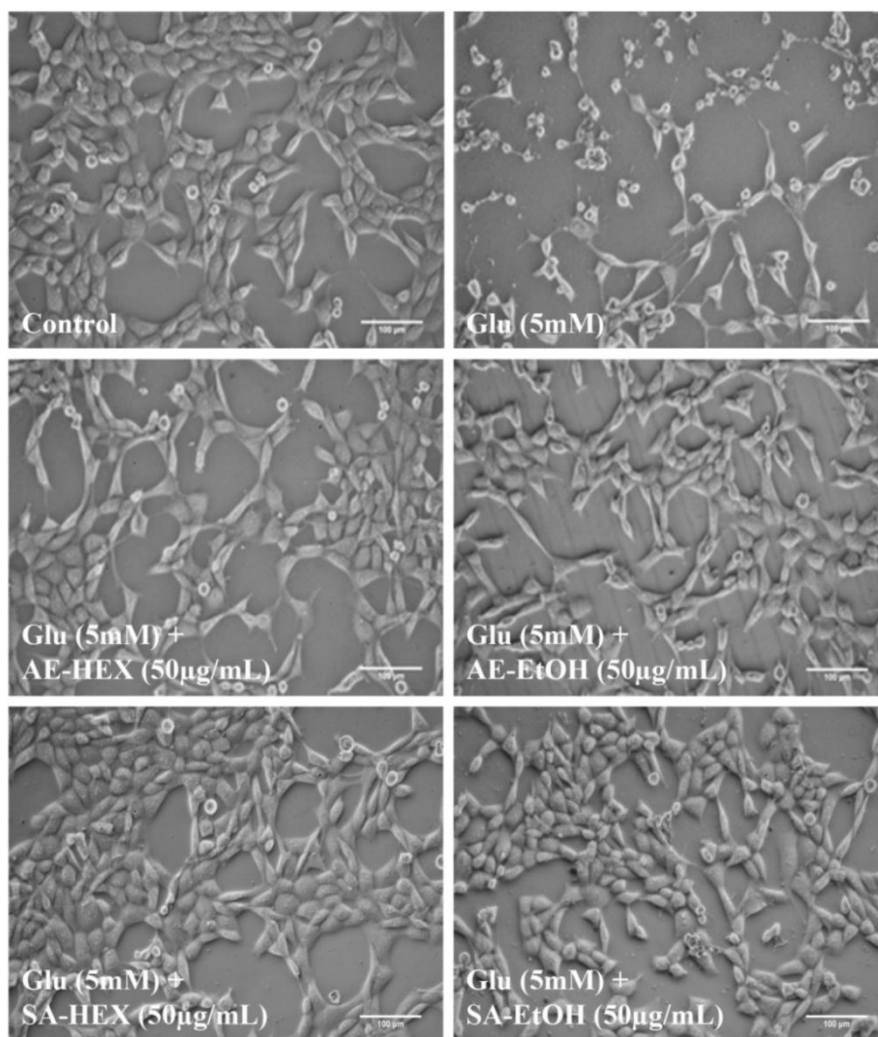


Figure 36: Representative morphological images showing the protective effects of *Acanthus ebracteatus* (AE) leaf extracts and *Streblus asper* (SA) leaf extracts on glutamate-treated HT-22 cells.

HT-22 cells were exposed to 5 mM glutamate alone or 5 mM glutamate combined with two different (hexane and ethanol) AE leaf extracts or two different (hexane and ethanol) SA leaf extracts at the concentration of 50 µg/mL for 24 h. Cell morphology was observed under a phase-contrast microscope at 5X magnification (scale bar = 100 µm).



#### 4.1.3 Investigation for the protective effect of Thai medicinal plant extracts against glutamate-induced apoptotic cell death

##### 4.1.3.1 Effects of Thai medicinal plant extracts against glutamate-induced apoptosis in HT-22 cells

To further clarify the neuroprotective effects of the ethanol extracts of *Acanthus ebracteatus* (AE) and *Streblus asper* (SA) leaf against glutamate-induced cytotoxicity, we examined whether both extracts could suppress glutamate-induced apoptotic cell death, since it is well known that the toxicity caused by excessive glutamate contributes to neuronal cell death via the apoptotic pathway [40]. The percentage of apoptotic death of HT-22 cells exposed to 5 mM glutamate in the presence or absence of AE and SA ethanol leaf extracts at the concentration of 50 µg/mL was quantified using the Annexin V-FITC/PI staining and flow cytometric analysis. Our results demonstrated the induction of apoptosis in HT-22 cells following glutamate exposure and that the percentage of apoptotic cell death in 5 mM glutamate-treated cells was dramatically increased to approximately 50% compared to that of the control, in which the majority of apoptotic cells were in late stage (Figure 37A). However, co-treatment of the cells with 50 µg/mL of AE or SA ethanol leaf extracts could significantly reduce the apoptotic rate of glutamate-treated cells to an extent comparable to that observed in the control cells (Figure 37B), indicating the cytoprotective and antiapoptotic effects of both extracts against glutamate toxicity in neurons.



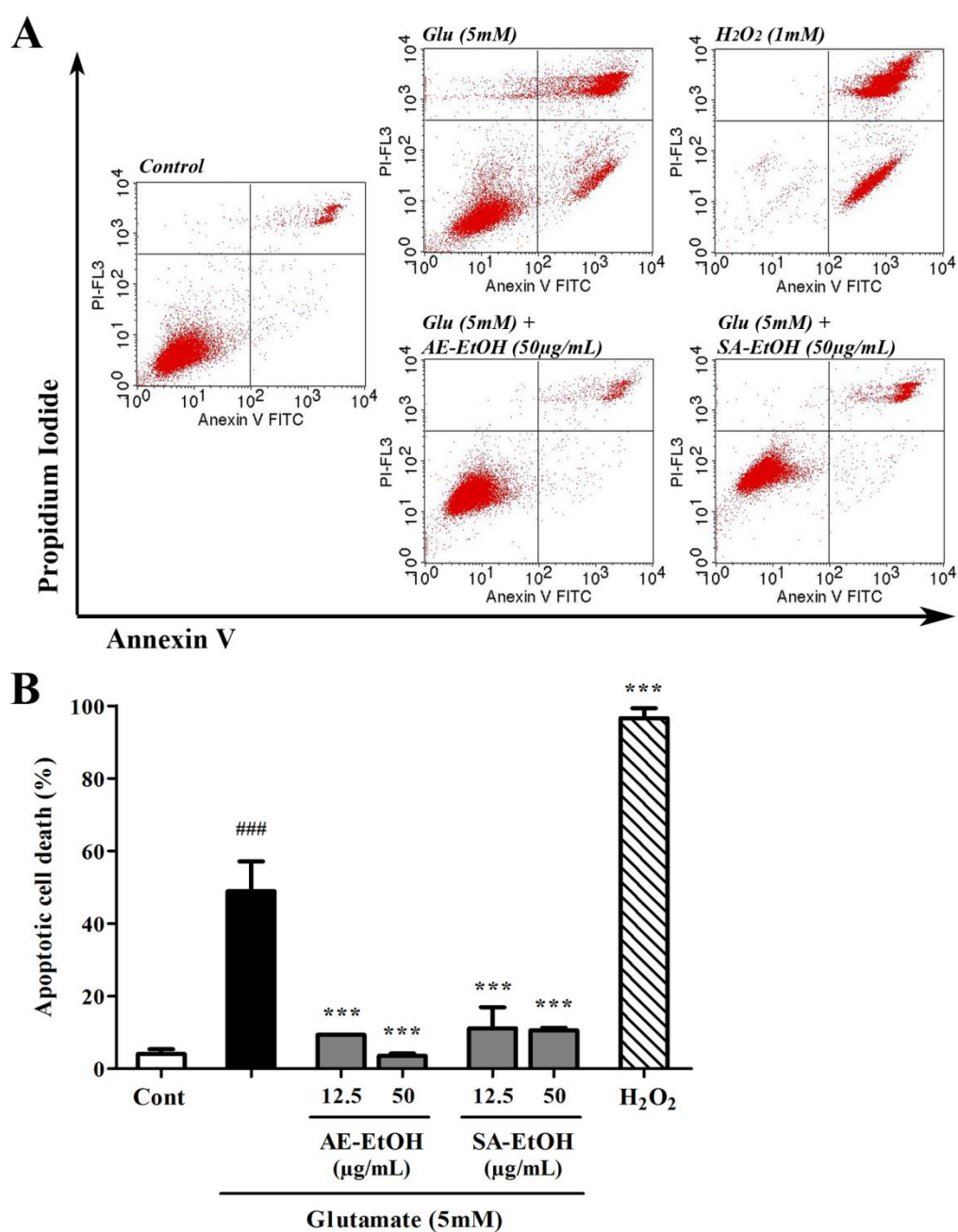


Figure 37: Protective effect of ethanol extracts of *Acanthus ebracteatus* (AE) and *Streblus asper* (SA) leaf against glutamate-induced apoptosis in HT-22 cells determined by flow cytometric analysis with annexin-V/PI staining.

(A) Representative flow cytometric scatter plots of annexin V-FITC and PI staining. HT-22 cells were exposed to 5 mM glutamate alone or 5 mM glutamate combined with either AE or SA ethanol leaf extracts at the concentration of 12.5 and 50 µg/mL for 18 h. HT-22 cells



exposed to  $\text{H}_2\text{O}_2$  (1 mM) were used as positive control. (B) The percentages of apoptotic cells in each group calculated as the sum of annexin V-positive/PI-negative cells (early-stage apoptosis, lower right quadrant) plus annexin V/PI double-positive cells (late-stage apoptosis, upper right quadrant). Data are expressed as the means  $\pm$  SD of at least duplicate experiments.  $^{###}P < 0.001$  vs. untreated cells (control);  $^{***}P < 0.001$  vs. glutamate treatment alone.

#### 4.1.3.2 Effects of Thai medicinal plant extracts against glutamate-induced nuclear translocation of apoptosis-inducing factor (AIF) in HT-22 cells

Nuclear translocation of apoptosis-inducing factor (AIF) is the major downstream mechanism underlying glutamate-induced neuronal cell death mediated through ROS formation. Elevated intracellular ROS levels caused by an excess of glutamate can induce the release of mitochondrial AIF to the nucleus, thereby triggering apoptosis in a caspase-independent manner [44]. Thus, we further examined whether the ethanol extracts of *Acanthus ebracteatus* (AE) and *Streblus asper* (SA) leaf could inhibit glutamate-induced AIF nuclear translocation. The immunofluorescence and Western blot analysis were performed in HT-22 cells treated with 5 mM glutamate in the presence or absence of AE and SA ethanol leaf extracts at the concentration of 50  $\mu\text{g/mL}$  to determine the effect of both extracts on the subcellular distribution of AIF. Our immunofluorescence results revealed that AIF proteins, which are mainly distributed throughout the cytosol under control conditions, were translocated into neuronal nuclei of the HT-22 cells following treatment with 5 mM glutamate (Figure 38). Moreover, the AIF expression detected by Western blotting was found to be significantly increased in the nucleus but decreased in the cytoplasm (Figure 39), confirming that glutamate caused the nuclear translocation of AIF. However, exposure of glutamate-treated cells to 50  $\mu\text{g/mL}$  of AE and SA ethanol leaf extracts significantly restored both nuclear and cytoplasmic expression of AIF proteins to a level similar to those of control cells (Figure 38 and 39). These results suggest that the



protective effect of both extracts against neuronal cell death may be mediated by the inhibition of glutamate-induced translocation of AIF to the nucleus.

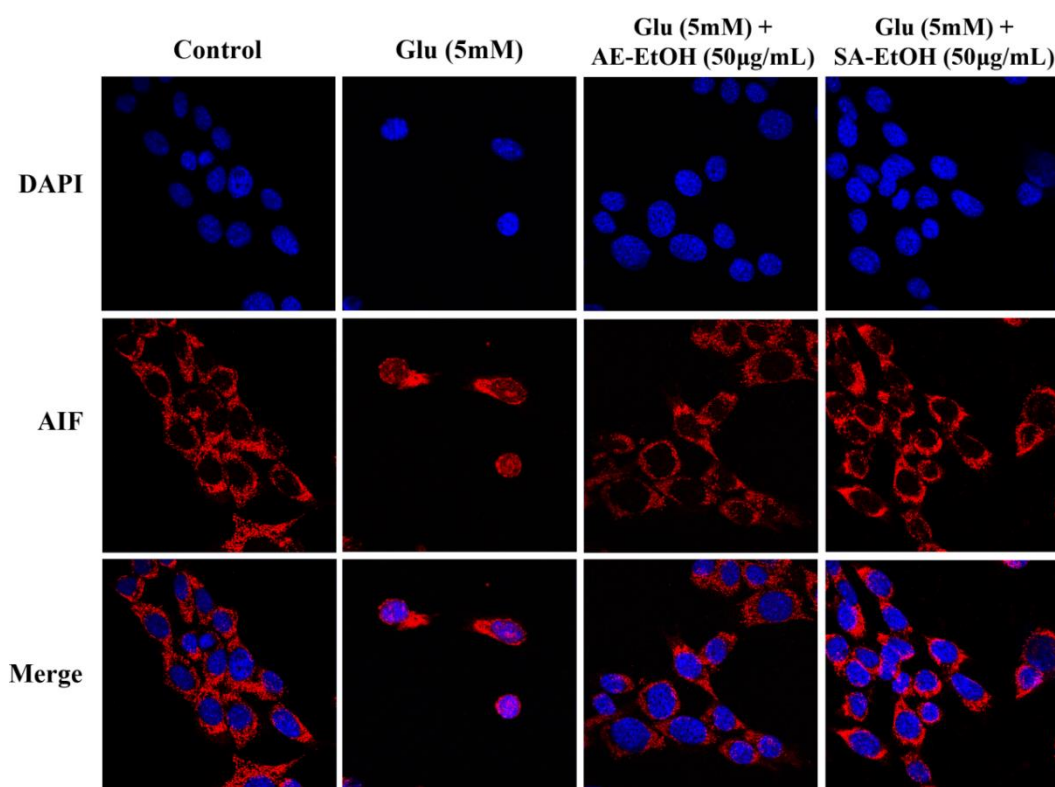


Figure 38: Representative confocal photographs showing the protective effect of ethanol extracts of *Acanthus ebracteatus* (AE) and *Streblus asper* (SA) leaf against glutamate-induced nuclear translocation of AIF in HT-22 cells determined by immunofluorescence.

HT-22 cells were exposed to 5 mM glutamate alone or 5 mM glutamate combined with either AE or SA ethanol leaf extracts at the concentration of 50 µg/mL for 16 h, followed by staining with a fluorescent-labeled antibody specific for AIF (red) and counterstaining with DAPI (blue) to indicate nuclear location.



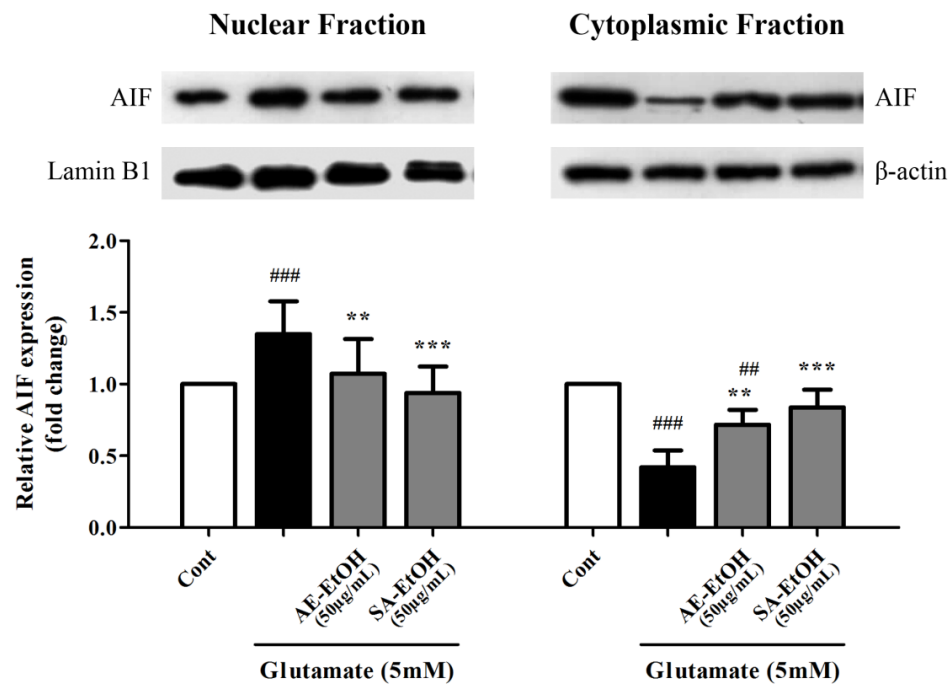


Figure 39: Protective effect of ethanol extracts of *Acanthus ebracteatus* (AE) and *Streblus asper* (SA) leaf against glutamate-induced nuclear translocation of AIF in HT-22 cells determined by Western blotting.

HT-22 cells were exposed to 5 mM glutamate alone or 5 mM glutamate combined with either AE or SA ethanol leaf extracts at the concentration of 50  $\mu$ g/mL for 16 h, followed by Western blot analysis of nuclear and cytoplasmic AIF levels. Lamin B1 and  $\beta$ -actin served as endogenous loading controls for nuclear extracts and whole cell/cytoplasmic extracts, respectively. All data were normalized to endogenous control levels and are expressed as the means  $\pm$  SD of at least three independent experiments.  $^{##}P < 0.01$ ,  $^{###}P < 0.001$  vs. untreated cells (control);  $^{**}P < 0.01$ ,  $^{***}P < 0.001$  vs. glutamate treatment alone.



#### 4.1.4 Investigation for the protective effect of Thai medicinal plant extracts against glutamate-induced oxidative stress

Enhanced oxidative stress and excessive ROS generation have been considered a pivotal mechanism which is underlying the cytotoxic action of glutamate at high concentration that eventually leads to neuronal cell death via AIF-mediated apoptosis [43, 44]. Thus, we further examined whether the ethanol extracts of *Acanthus ebracteatus* (AE) and *Streblus asper* (SA) leaf could inhibit glutamate-induced oxidative stress. Antioxidant capacities of the AE and SA ethanol leaf extracts were indirectly evaluated by determining the amount of phenolic and flavonoid compounds. Moreover, the antioxidant activities of both extracts were directly evaluated through *in vitro* free radical scavenging assays and cell-based antioxidant assay.

##### 4.1.4.1 Total phenolic content, total flavonoid content, and *in vitro* antioxidant capacities of Thai medicinal plant extracts

Phenolics are the major group of phytochemical compounds which most of them naturally possess antioxidant properties [381]. The general antioxidant mechanism of phenolics relies on their electron and/or hydrogen atom donating abilities, thereby inhibiting the formation of or interrupting the propagation of free radicals [382, 383]. Thus, the amount of phenolic compounds including flavonoids which comprise the most abundant class of plant phenolics, were determined as an indicator of the antioxidant content of the plant. Table 5 shows the total phenolic and total flavonoid contents of five Thai medicinal plants used in the present study. Total phenolic contents were varied widely in the different plant extracts and ranged from  $3.44 \pm 0.92$  to  $460.25 \pm 3.08$  mg GAE/g dry weight. Whereas, total flavonoid contents were much lower with less variation ranging from  $0.78 \pm 0.40$  to  $20.22 \pm 3.69$  mg QE/g dry weight. Amongst twelve extracts of five plants, the ethanol extracts of AE and SA leaf presented the third and the fifth-highest total phenolic contents as well as the highest and the fourth-highest total flavonoid contents. To directly examine the antioxidant capacities via hydrogen atom- and/or



electron-donating mechanisms, five Thai medicinal plants were evaluated *in vitro* for their scavenging activities against the DPPH and ABTS free radicals. At the extract concentration of 1 mg/ml, the DPPH and ABTS free radical scavenging activities varied greatly from 0.05% to 95.50% ( $0.67 \pm 0.77$  to  $808.41 \pm 9.65$  mg VCEAC/g dry weight) (Table 6), and from 3.96% to 99.71% ( $2.75 \pm 1.03$  to  $1140.49 \pm 19.10$  mg VCEAC/g dry weight) (Table 7), respectively. The ranking of the plant extracts according to their DPPH and ABTS free radical scavenging activities were found similar to the ranking by their total phenolic contents. Overall, the methanol extract of *Caesalpinia mimosoides* (CM) twigs and leaf exhibited the highest *in vitro* antioxidant activity and possesses a higher amount of total phenolic and flavonoid contents compared to others. The abilities of AE ethanol leaf extract to scavenge DPPH and ABTS free radicals, as well as its total phenolic and flavonoid contents, were higher than those of SA ethanol leaf extract.

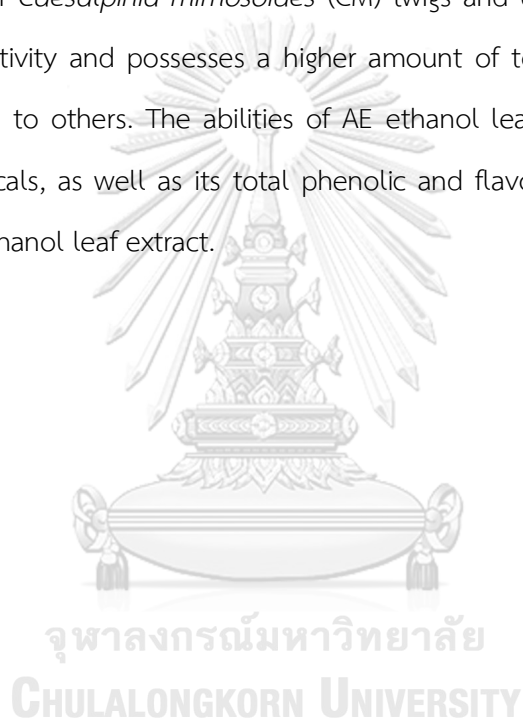




Table 5: Total phenolic and total flavonoid contents of Thai medicinal plant extracts.

Plant	Solvent	Total phenolic content (mg GAE/g dry weight sample)	Total flavonoid content (mg QE/g dry weight sample)
<i>Morinda citrifolia</i> (MC)	Hexane	4.68 ± 0.85	0.98 ± 0.16
	Ethyl acetate	35.74 ± 1.91	1.51 ± 0.20
	Methanol	12.58 ± 0.59	1.84 ± 0.77
<i>Caesalpinia mimosoides</i> (CM)	Hexane	5.35 ± 0.85	1.76 ± 0.32
	Ethyl acetate	323.21 ± 6.45	8.89 ± 0.39
	Methanol	460.25 ± 3.08	12.55 ± 0.43
<i>Murdannia loriformis</i> (ML)	Hexane	3.44 ± 0.92	0.78 ± 0.40
	Ethanol	17.12 ± 1.43	2.93 ± 0.65
<i>Acanthus ebracteatus</i> (AE)	Hexane	4.07 ± 1.09	1.09 ± 1.42
	Ethanol	84.86 ± 1.68	20.22 ± 3.69
<i>Streblus asper</i> (SA)	Hexane	4.15 ± 1.21	0.82 ± 0.23
	Ethanol	26.89 ± 0.96	5.17 ± 1.17

GAE is gallic acid equivalent; QE is quercetin equivalent

Values are expressed as mean ± SD of at least three replicates.



Table 6: Free radical scavenging activities of Thai medicinal plant extracts determined by the DPPH scavenging assay.

Plant	Solvent	DPPH radical scavenging activity <sup>a</sup> (% Inhibition)	mg VCEAC/g dry weight sample
<i>Morinda citrifolia</i> (MC)	Hexane	2.45 ± 0.74	3.34 ± 0.82
	Ethyl acetate	16.04 ± 0.43	18.46 ± 1.03
	Methanol	4.63 ± 0.60	5.78 ± 1.07
<i>Caesalpinia mimosoides</i> (CM)	Hexane	1.33 ± 0.40	2.09 ± 0.26
	Ethyl acetate	95.50 ± 0.06	466.17 ± 7.14
	Methanol	94.03 ± 0.10	808.41 ± 9.65
<i>Murdannia loriformis</i> (ML)	Hexane	0.05 ± 0.82	0.67 ± 0.77
	Ethanol	4.47 ± 0.34	5.58 ± 0.38
<i>Acanthus ebracteatus</i> (AE)	Hexane	1.65 ± 0.17	2.44 ± 0.31
	Ethanol	58.46 ± 0.76	65.60 ± 1.21
<i>Streblus asper</i> (SA)	Hexane	2.25 ± 1.00	3.11 ± 0.81
	Ethanol	15.38 ± 0.70	17.70 ± 0.39

<sup>a</sup>Percentage inhibition of the extract at the concentration of 1 mg/mL

VCEAC is vitamin C equivalent antioxidant capacity

Values are expressed as mean ± SD of at least three replicates.



Table 7: Free radical scavenging activities of Thai medicinal plant extracts determined by the ABTS scavenging assay.

Plant	Solvent	ABTS radical scavenging activity <sup>a</sup> (% Inhibition)	mg VCEAC/g dry weight sample
<i>Morinda citrifolia</i> (MC)	Hexane	7.62 ± 1.02	4.65 ± 0.60
	Ethyl acetate	49.74 ± 6.16	26.97 ± 3.47
	Methanol	18.22 ± 2.06	10.27 ± 1.34
<i>Caesalpinia mimosoides</i> (CM)	Hexane	6.47 ± 0.50	4.11 ± 0.56
	Ethyl acetate	99.71 ± 0.02	461.79 ± 6.53
	Methanol	99.56 ± 0.07	1140.49 ± 19.10
<i>Murdannia loriformis</i> (ML)	Hexane	3.96 ± 1.83	2.75 ± 1.03
	Ethanol	24.53 ± 2.26	13.99 ± 1.44
<i>Acanthus ebracteatus</i> (AE)	Hexane	7.29 ± 1.36	4.54 ± 0.87
	Ethanol	89.98 ± 6.83	72.01 ± 1.02
<i>Streblus asper</i> (SA)	Hexane	8.12 ± 0.54	4.98 ± 0.46
	Ethanol	56.5 ± 6.28	31.03 ± 3.98

<sup>a</sup>Percentage inhibition of the extract at the concentration of 1 mg/mL

VCEAC is vitamin C equivalent antioxidant capacity

Values are expressed as mean ± SD of at least three replicates.



#### 4.1.4.2 Effects of Thai medicinal plant extracts against glutamate-induced oxidative stress in HT-22 cells

Cell-based antioxidant assay was performed using 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA) probe to evaluate oxidative stress status and the extent of ROS inside the cell. Prior to beginning the experiment, we first determined the optimal treatment time of glutamate to achieve the maximum amount of fluorescent signal. Treatment of HT-22 cells with 5 mM glutamate for various times ranging from 0 to 18 h resulting in increased DCF-derived fluorescence intensity upon glutamate treatment up to 14 h, and after that the signal was decreased (Figure 40). Thus, 14 h was chosen as the optimal treatment time of glutamate for inducing oxidative stress in HT-22 cells. The experiments were then performed in HT-22 cells treated with 5 mM glutamate for 14 h in the presence or absence of AE and SA ethanol extracts at different concentrations to determine the antioxidant activities of both extracts. The results revealed that 5 mM glutamate treatment caused significantly increased intracellular oxidative stress and ROS accumulation in HT22 cells, as represented by an approximately two-fold higher DCF-derived fluorescence intensity relative to untreated cells (control) (Figure 41A and 41B). However, exposure of glutamate-treated cells to different concentrations of AE and SA ethanol leaf extracts significantly decreased intracellular ROS accumulation in a dose-dependent manner, which the extracts at a concentration 50 µg/mL were able to restore ROS levels to that of control (Figure 41B). These results suggest that the protective effect of both extracts against glutamate-induced neuronal cell death may be mediated by suppressing intracellular oxidative stress.



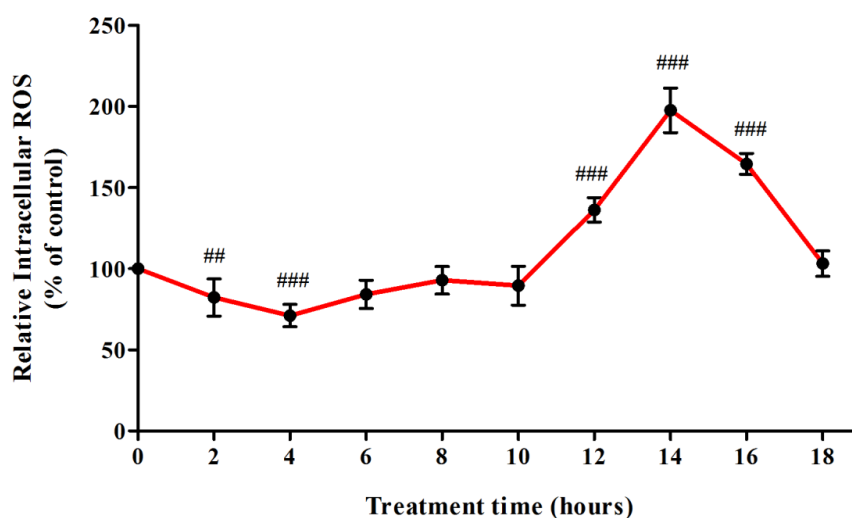


Figure 40: Effect of glutamate on the intracellular ROS generation in HT-22 cells determined by the DCFH-DA method.

HT-22 cells were exposed to glutamate at a concentration of 5 mM for various exposure times, followed by staining with DCFH-DA probe. Data are expressed as the means  $\pm$  SD of at least three independent experiments. <sup>##</sup> $P < 0.01$ , <sup>###</sup> $P < 0.001$  vs. untreated cells (control).



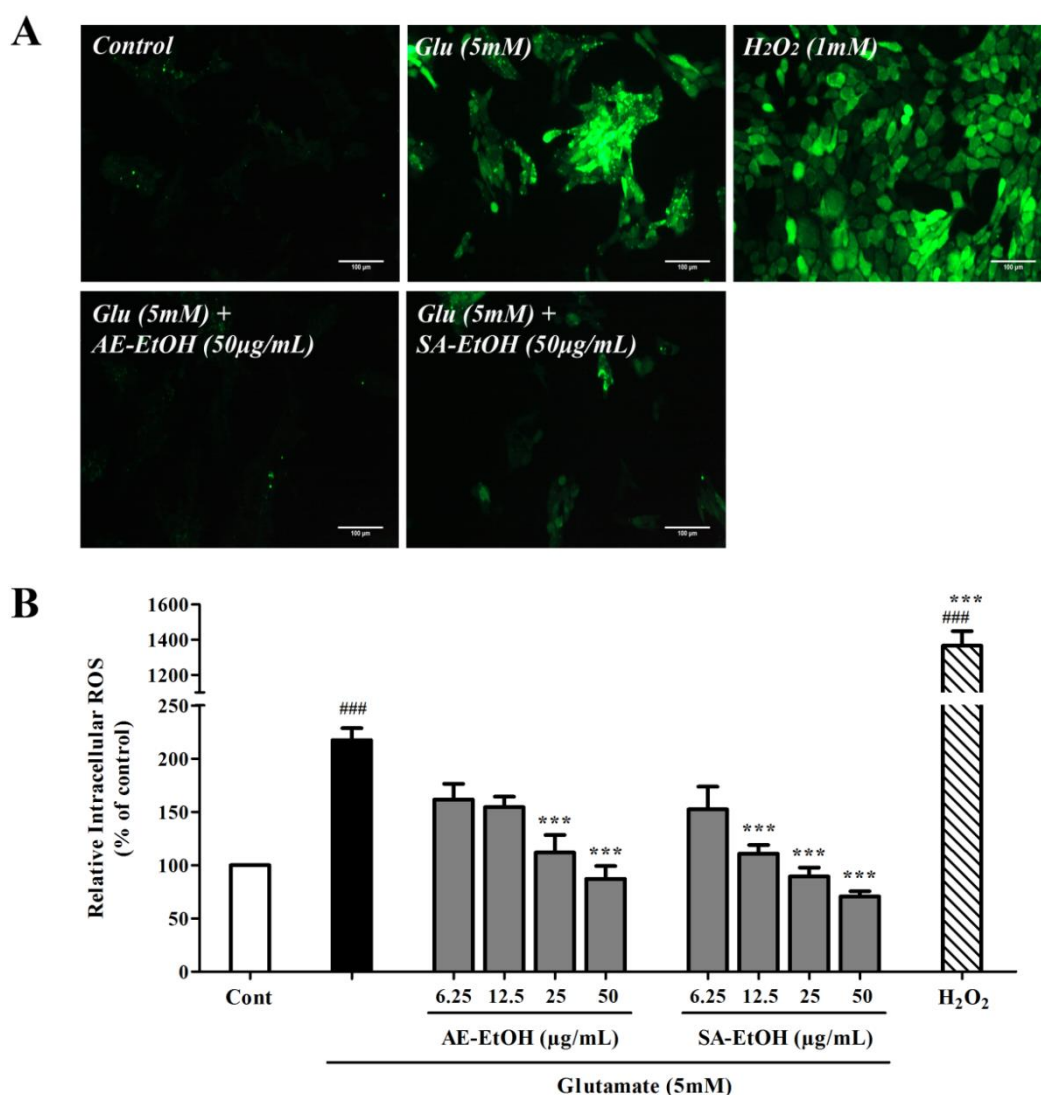


Figure 41: Protective effect of ethanol extracts of *Acanthus ebracteatus* (AE) and *Streblus asper* (SA) leaf against glutamate-induced intracellular ROS generation in HT-22 cells determined by the DCFH-DA method.

HT-22 cells were exposed to 5 mM glutamate alone or 5 mM glutamate combined with a varied concentration of either AE or SA ethanol leaf extracts for 14 h. HT-22 cells exposed to H<sub>2</sub>O<sub>2</sub> (1 mM) were used as positive control. (A) Representative fluorescence micrographs of the cells stained with DCFH-DA probe observed under a fluorescence microscope (scale bar = 100 µm). (B) Relative intracellular ROS levels quantified using a fluorescent plate reader. Data are expressed as the means ± SD of at least three independent experiments.

###  $P < 0.001$  vs. untreated cells (control); \*\*\* $P < 0.001$  vs. glutamate treatment alone.



#### 4.1.4.3 Effects of Thai medicinal plant extracts on Nrf2 pathway activation in HT-22 cells

It is well known that the induction of the nuclear factor erythroid 2-related factor 2 (Nrf2)/antioxidant response element (ARE) signaling pathway is a major mechanism of cellular protection against oxidative stress by controlling the expression of antioxidant-related genes whose protein products are involved in the elimination of free radicals [384, 385]. Thus, we further examined the effects of ethanol extracts of *Acanthus ebracteatus* (AE) and *Streblus asper* (SA) leaf on the Nrf2 signaling pathway in order to identify the mechanism of action of both extracts in antioxidant-mediated neuroprotection against glutamate toxicity. Real-time RT-PCR and Western blot analysis were performed in HT-22 cells treated with 5 mM glutamate in the presence or absence of AE and SA ethanol leaf extracts at the concentration of 50 µg/mL. Our Western blot results revealed that exposure of glutamate-treated cells to 50 µg/mL of AE and SA ethanol leaf extracts caused rapid nuclear accumulation of transcription factor Nrf2 without altering its cytoplasmic level. The level of Nrf2 expression in the nucleus was found significantly increased by 2.7- and 3.5-fold the level of the control after an hour-long treatment of HT-22 cells with glutamate and AE and SA ethanol leaf extracts, respectively (Figure 42), indicating activation of Nrf2. In addition, both extracts significantly increased the mRNA expression levels of antioxidant-related genes under Nrf2 regulation, including excitatory amino acid transporter 3 (*EAAT3*), NAD(P)H:quinone oxidoreductase (*NQO1*), and glutamate-cysteine ligase modifier subunit (*GCLM*), by approximately 3- to 4-fold over the control level (Figure 43). These findings were also correlated with a significant increase in protein expression of *EAAT3*, which was confirmed by Western blot analysis (Figure 44). In parallel, we also investigated the effect of curcumin, a naturally occurring phenolic compound of turmeric with strong antioxidant activity, on the Nrf2 signaling pathway. Exposure of glutamate-treated cells to 15 µM curcumin resulted in a significant increase in transcriptional expression of *NQO1* and *GCLM*, whereas the expression of *EAAT3* mRNA did not change (Figure 43). Collectively, these findings demonstrate that both extracts promote antioxidant



defense by activating transcription factor Nrf2 and the expression of downstream antioxidant-related genes of the Nrf2/ARE signaling pathway, providing a plausible mechanism for their protective effects against glutamate-induced neuronal cell death.

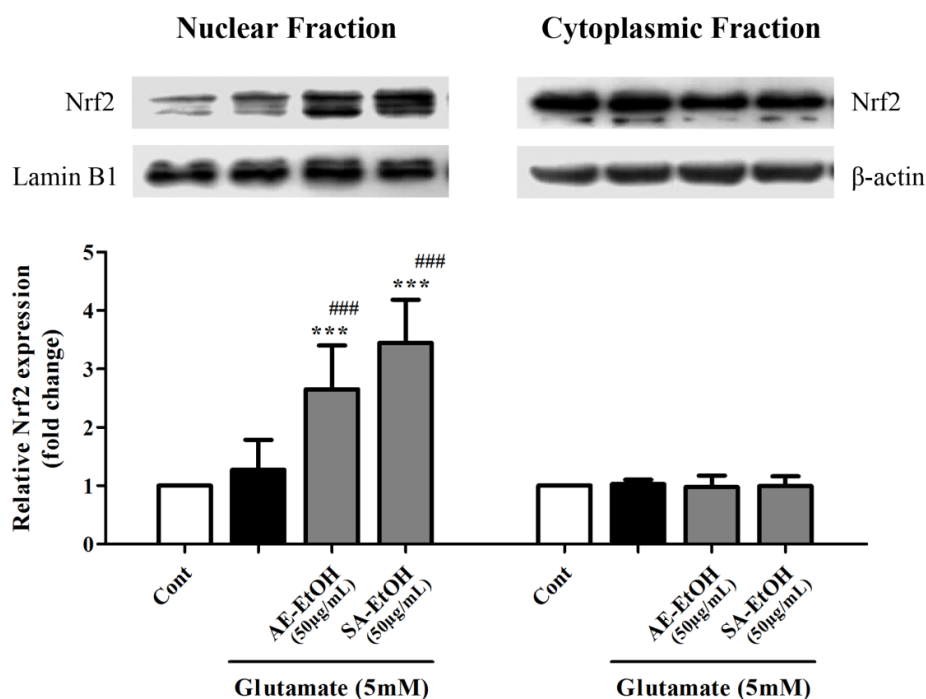


Figure 42: Effect of ethanol extracts of *Acanthus ebracteatus* (AE) and *Streblus asper* (SA) leaf on nuclear translocation of Nrf2 in glutamate-treated HT-22 cells determined by Western blotting.

HT-22 cells were exposed to 5 mM glutamate alone or 5 mM glutamate combined with either AE or SA ethanol leaf extracts at the concentration of 50 µg/mL for 1 h, followed by Western blot analysis of nuclear and cytoplasmic Nrf2 levels. Lamin B1 and β-actin served as endogenous loading controls for nuclear extracts and whole cell/cytoplasmic extracts, respectively. All data were normalized to endogenous control levels and are expressed as the means ± SD of at least three independent experiments. <sup>###</sup> $P < 0.001$  vs. untreated cells (control); <sup>\*\*\*</sup> $P < 0.001$  vs. glutamate treatment alone.



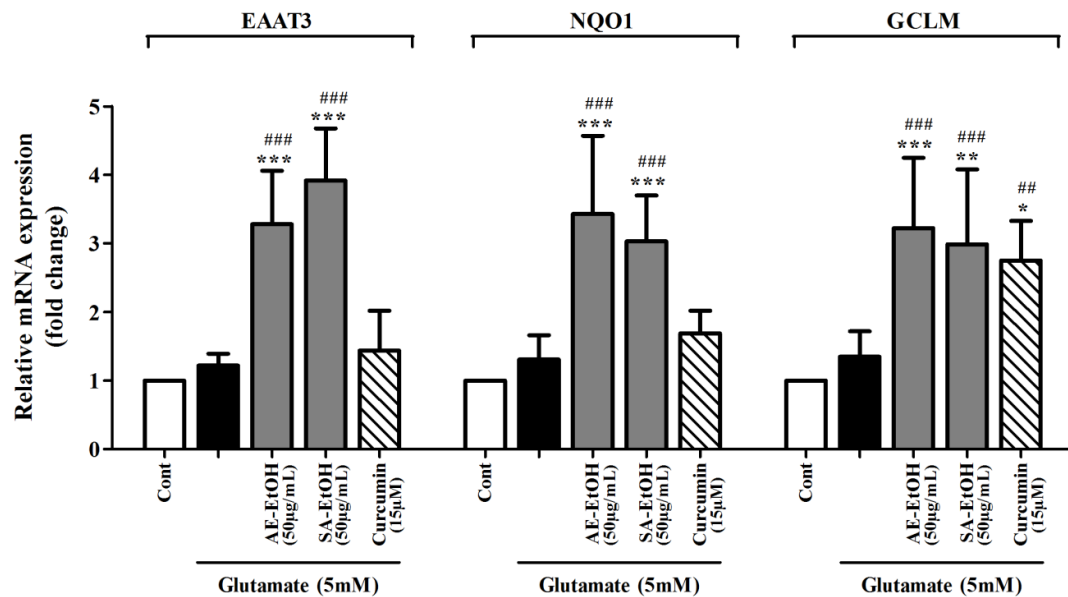


Figure 43: Effect of ethanol extracts of *Acanthus ebracteatus* (AE) and *Streblus asper* (SA) leaf on expression of Nrf2-regulated antioxidant genes (*EAAT3*, *NQO1*, and *GCLM*) in glutamate-treated HT-22 cells determined by quantitative real-time RT-PCR analysis.

HT-22 cells were exposed to 5 mM glutamate alone or 5 mM glutamate combined with either AE or SA ethanol leaf extracts at the concentration of 50 µg/mL for 24 h. HT-22 cells exposed to curcumin (15 µM) were used as positive control.  $\beta$ -actin (*ACTB*) served as an internal control to normalize the mRNA expression levels. Data are expressed as the means  $\pm$  SD of at least three independent experiments.  $^{##}P < 0.01$ ,  $^{###}P < 0.001$  vs. untreated cells (control);  $^{*}P < 0.05$ ,  $^{**}P < 0.01$ ,  $^{***}P < 0.001$  vs. glutamate treatment alone.



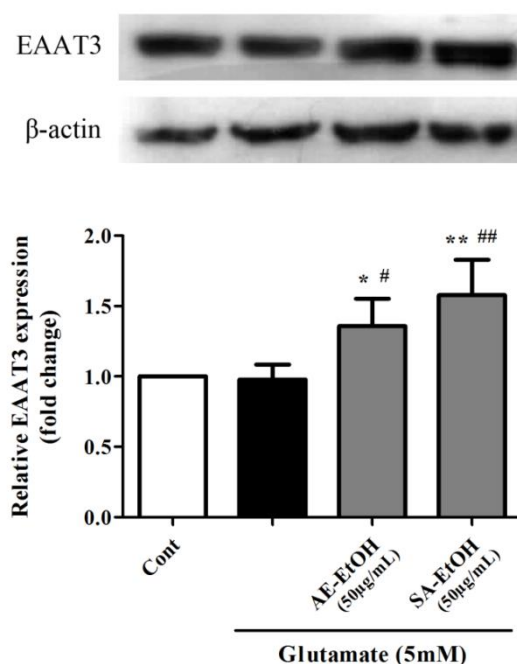


Figure 44: Effect of ethanol extracts of *Acanthus ebracteatus* (AE) and *Streblus asper* (SA) leaf on protein expression of EAAT3 in glutamate-treated HT-22 cells determined by Western blotting.

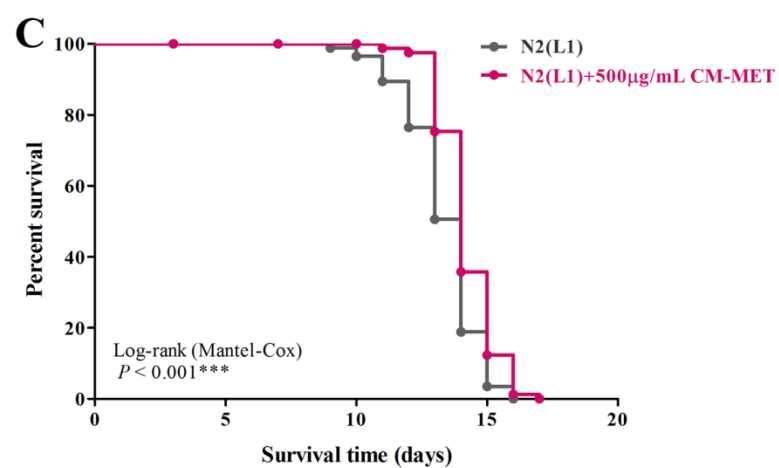
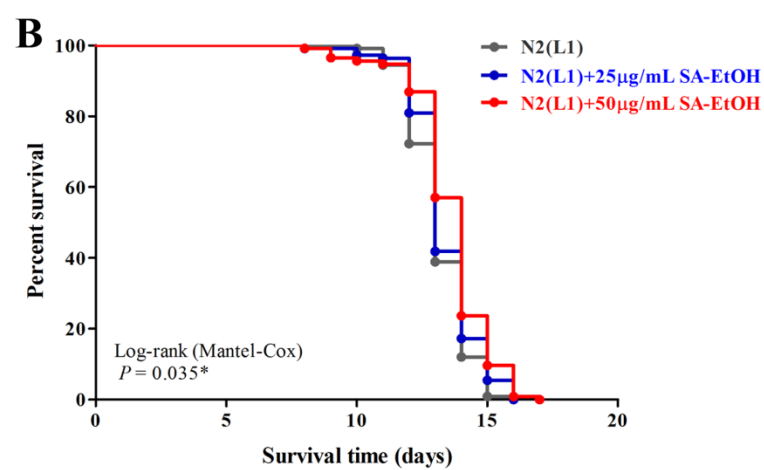
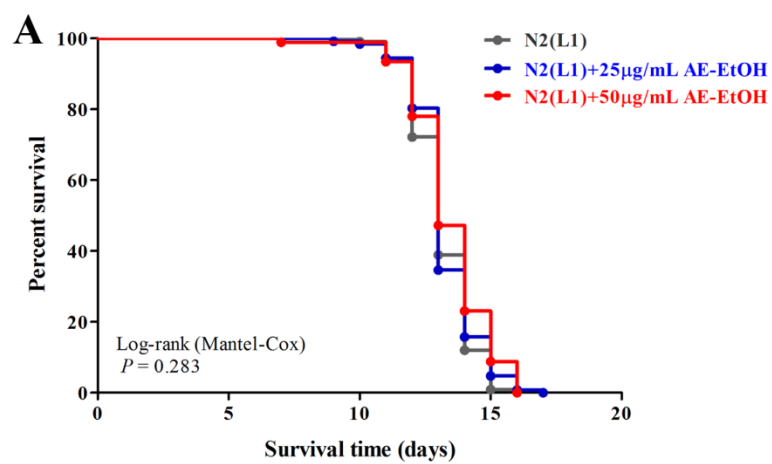
HT-22 cells were exposed to 5 mM glutamate alone or 5 mM glutamate combined with either AE or SA ethanol leaf extracts at the concentration of 50  $\mu$ g/mL for 24 h, followed by Western blot analysis of EAAT3 levels in whole cell lysates.  $\beta$ -actin served as an endogenous loading control. All data were normalized to endogenous control levels and are expressed as the means  $\pm$  SD of at least three independent experiments. <sup>#</sup> $P < 0.05$ , <sup>##</sup> $P < 0.01$  vs. untreated cells (control); <sup>\*</sup> $P < 0.05$ , <sup>\*\*</sup> $P < 0.01$  vs. glutamate treatment alone.



#### 4.1.5 Investigation for the longevity effect of Thai medicinal plant extracts in *C. elegans*

*Acanthus ebracteatus* (AE) and *Streblus asper* (SA) have long been recognized as ingredients in the popular Thai traditional formulas for longevity [320, 355], however, their longevity effects have never been explored in the scientific literature. Thus, in the present study, lifespan extension activities of AE and SA were directly evaluated using the nematode *C. elegans* as an *in vivo* model. *Caesalpinia mimosoides* (CM) was also selected to investigate for its effect on the lifespan of *C. elegans* because this plant possessed the highest amount of total phenolic and flavonoid contents and showed the highest *in vitro* antioxidant activity amongst the studied plants. Lifespan assays were performed in *C. elegans* Bristol N2 strain at L1 or L4 larval stage fed with *E. coli* OP50 supplemented with different concentrations of AE and SA ethanol leaf extracts as well as CM methanol twig and leaf extract. The results revealed that AE ethanol leaf extract did not extend the lifespan of *C. elegans* Bristol N2 at both larval stages (Figure 45A and 45D). However, we found that SA ethanol leaf extract was capable of enhancing survival of *C. elegans* Bristol N2 at L1 larval stage (Figure 45B), but not the L4 larval stage (Figure 45E), with the significant difference of survival rates among the groups at  $P < 0.05$ . The mean lifespan of the SA ethanol leaf extract-treated L1-stage worms was 13.64 days that was slightly but significantly increased when compared to the control worms (Table 8). Interestingly, CM methanol twig and leaf extract could substantially influence the survival of the worms at both L1 and L4 larval stages, with the significant difference of survival rates among the groups at  $P < 0.001$  (Figure 45C and 45F) as well as the significant increase of mean lifespan by 6% in L1 stage worms and by 10% at L4 stage worms (Table 8). These results suggest that the ethanol extract of AE leaf and the methanol extract of CM twigs and leaf exert lifespan-extending properties in *C. elegans* Bristol N2 strain.







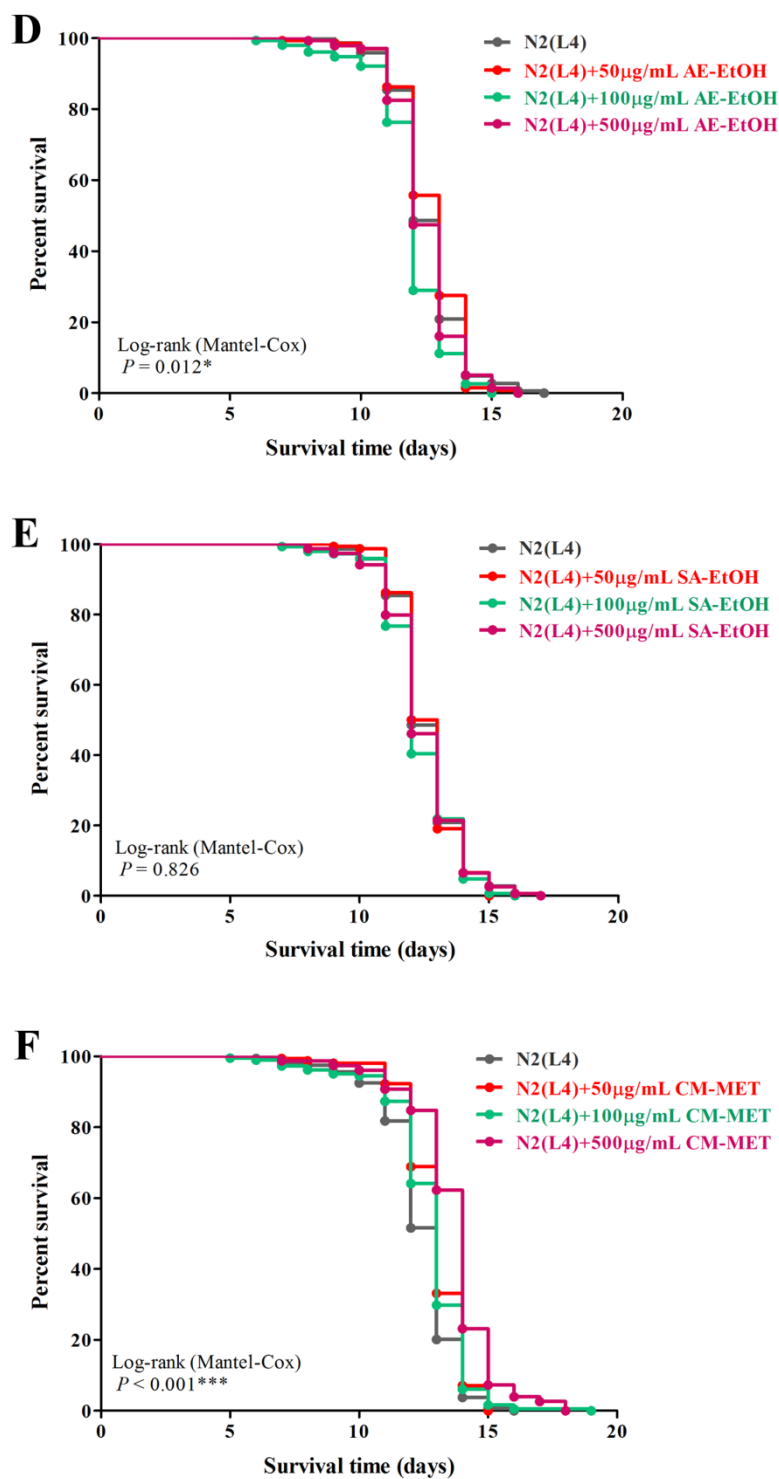


Figure 45: Effect of Thai medicinal plant extracts on the lifespan of *C. elegans*.

Cumulative survival plots of *C. elegans* Bristol N2 strain at L1 (A-C) and L4 larval stages (D-F) grown at 25°C treated with different concentrations of *Acanthus ebracteatus* (AE)



ethanol extract (A and D), or *Streblus asper* (SA) ethanol extract (B and E), or *Caesalpinia mimosoides* (CM) methanol extract (C and F), or DMSO in control group. All data are shown as the means of three independent experiments. The statistical comparison of survival curves was performed with Log-rank (Mantel-Cox) test; \* $P < 0.05$ , \*\*\* $P < 0.001$ .

Table 8: Results and statistical analyses of *C. elegans* lifespan assays.

Treatment	Number of worms	Mean lifespan (days)	Maximum lifespan (days)	Percentage of increased lifespan (vs control)	<i>P</i> value (vs control)
N2(L1)					
Control	108	13.18 ± 0.11	16	-	-
25 µg/mL AE-EtOH	127	13.28 ± 0.11	17	0.76	0.490
50 µg/mL AE-EtOH	91	13.46 ± 0.15	16	2.12	0.124
N2(L1)					
Control	108	13.18 ± 0.11	16	-	-
25 µg/mL SA-EtOH	110	13.37 ± 0.12	16	1.44	0.232
50 µg/mL SA-EtOH	114	13.64 ± 0.14	17	3.49	0.010*
N2(L1)					
Control	85	13.34 ± 0.15	16	-	-
500 µg/mL CM-MET	81	14.21 ± 0.12	17	6.52	<0.001***
N2(L4)					
Control	144	12.58 ± 0.11	17	-	-
50 µg/mL AE-EtOH	131	12.66 ± 0.11	16	0.64	0.598
100 µg/mL AE-EtOH	152	11.99 ± 0.12	15	-4.69	<0.001***
500 µg/mL AE-EtOH	137	12.47 ± 0.11	16	-0.87	0.467
N2(L4)					
Control	144	12.58 ± 0.11	17	-	-
50 µg/mL SA-EtOH	152	12.60 ± 0.09	15	0.16	0.873
100 µg/mL SA-EtOH	146	12.35 ± 0.12	16	-1.83	0.149
500 µg/mL SA-EtOH	154	12.47 ± 0.12	17	-0.87	0.516



N2(L4)					
Control	159	12.41 ± 0.12	16	-	-
50 µg/mL CM-MET	154	12.95 ± 0.10	15	4.35	<0.001***
100 µg/mL CM-MET	181	12.72 ± 0.13	19	2.50	0.078
500 µg/mL CM-MET	151	13.66 ± 0.14	18	10.07	<0.001***

AE = *Acanthus ebracteatus*; SA = *Streblus asper*; CM = *Caesalpinia mimosoides*

Mean lifespans were expressed as the means ± standard error of the mean (SEM) and compared by Student's t test; \* $P < 0.05$ , \*\*\* $P < 0.001$  vs control (two-tailed)

#### 4.1.6 Investigation for phytochemical constituents of Thai medicinal plant extracts

To identify putative phytochemical components in *Acanthus ebracteatus* (AE) and *Streblus asper* (SA) ethanol leaf extracts that may be responsible for neuroprotection against glutamate-induced toxicity and/or longevity in *C. elegans*, we carried out LC-MS analysis in both extracts. Then their chromatographic peaks were identified for candidate compounds based on the search of  $m/z$  values of molecular ion peaks in the positive mode  $[M+H]^+$  by comparison of observed  $m/z$  values with the calculated (theoretical) values recorded in databases and the literature. Our results revealed a total of 95 ion chromatographic peaks of AE ethanol leaf extract detected in the positive ion mode (Figure 46). After identification of each molecular ion peak, we proposed 11 phytochemical compounds that could have beneficial effects for antioxidant defense or neurological function, of which 5 (peak no. 13, 25, 31, 32, 35) were reported for AE (Figure 46). The identified peaks are annotated by number and detailed in Table 9 as follows: peak number, retention time (Rt), observed  $m/z$ , peak area, compound name, theoretical mass, mass error, and database or reference. In the prediction of candidate compounds in SA ethanol leaf extract, the LC-MS results revealed more than 70 isolated peaks in the total ion chromatogram (Figure 47), where the peaks of candidate compounds are annotated by number and detailed in Table 10. In this study, we report four phytochemical compounds (peak no. 22, 42, 59, 65) proposed as active ingredients with neuroprotective and/or



longevity properties along with three compounds (peak no. 47, 55, 67) that were previously reported for SA.

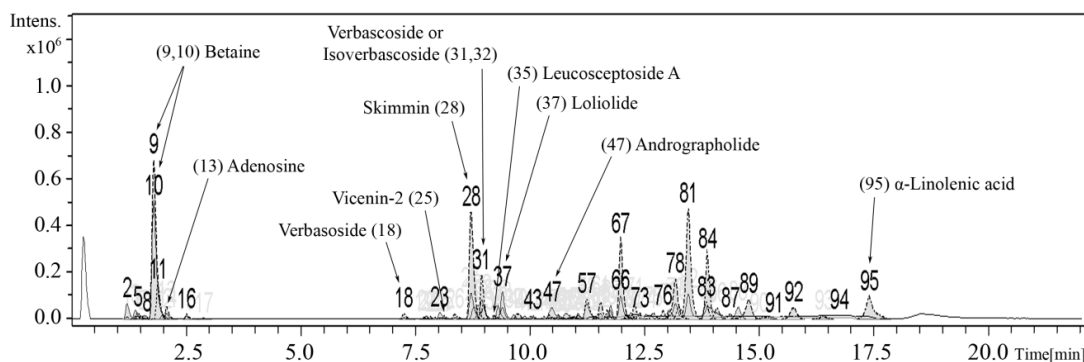


Figure 46: LC-MS total ion chromatogram of *Acanthus ebracteatus* ethanol leaf extract obtained in positive ESI mode.

All indicated peak numbers of proposed compounds are detailed in Table 9.

Table 9: Proposed phytochemical constituents in *Acanthus ebracteatus* ethanol leaf extract.

Peak No.	Rt (min)	[M+H] <sup>+</sup> (m/z)	Area (%)	Proposed compound	Theoretical mass	Mass error (ppm)	Database/Reference
9	1.8	118.088	10.5	Betaine	117.079	14	METLIN
10	1.8	118.087	7.7	Betaine	117.079	6	METLIN
13	2.1	268.104	0.7	Adenosine	267.097	0	METLIN, [348]
18	7.3	463.181	0.4	Verbascoside	462.174	0	METLIN
25	8.1	595.165	0.2	Vicenin-2	594.158	1	METLIN, [348]
28	8.7	325.091	6.9	Skimmin	324.085	2	METLIN, KNApSack
31	9.0	625.212	2.3	Verbascoside, Isoverbascoside	624.205	1	METLIN, [348]
32	9.0	625.210	1.2	Verbascoside, Isoverbascoside	624.205	4	METLIN, [348]
35	9.2	639.228	0.2	Leucosceptoside A	638.221	1	[348]
37	9.4	197.117	1.7	Lolilide	196.110	1	METLIN, KNApSack



47	10.5	351.214	1.1	Andrographolide	350.209	7	METLIN
95	17.4	279.231	2.9	$\alpha$ -Linolenic Acid	278.225	3	METLIN

Rt = Retention time

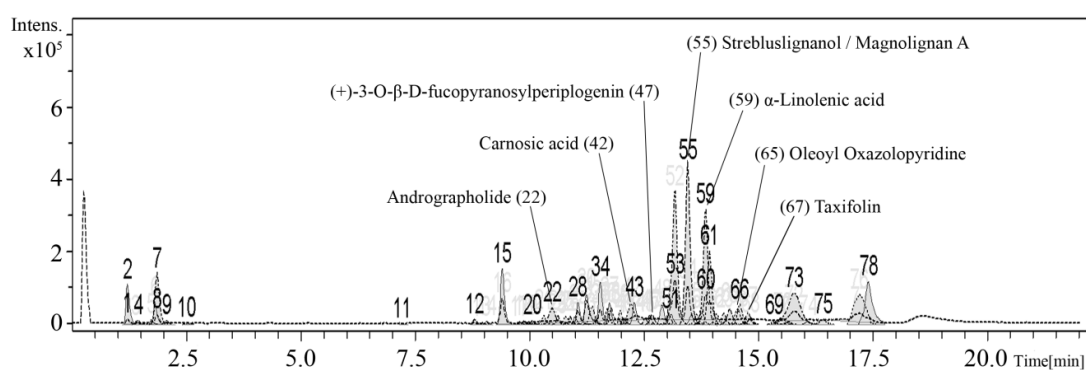


Figure 47: LC-MS total ion chromatogram of *Streblus asper* ethanol leaf extract obtained in positive ESI mode.

All indicated peak numbers of proposed compounds are detailed in Table 10.

Table 10: Proposed phytochemical constituents in *Streblus asper* ethanol leaf extract.

Peak No.	Rt (min)	[M+H] <sup>+</sup> (m/z)	Area (%)	Proposed compound	Theoretical mass	Mass error (ppm)	Database/Reference
22	10.5	351.215	1.4	Andrographolide	350.209	4	METLIN, KNApSACK
42	12.2	333.205	1.8	Carnosic acid	332.199	3	METLIN, KNApSACK
47	12.7	537.307	0.6	(+)-3-O-β-D-fucopyranosyl-periplogenin	536.298	3	[356]
55	13.5	301.141	9.8	Strebluslignanol, Magnolignan A	300.136	9	[386]
59	13.8	279.232	6.8	$\alpha$ -Linolenic acid	278.225	0	METLIN
65	14.5	385.292	1.1	Oleoyl Oxazolopyridine	384.277	19	METLIN
67	14.8	305.248	0.8	Taxifolin	304.250	30	[387]

Rt = Retention time



## 4.2 The study of bioactive properties of the acid-base fractions isolated from *Streblus asper* leaf extract

### 4.2.1 Extraction yield of the acid-base fractions isolated from *Streblus asper* leaf extract

The ethanol extract of *Streblus asper* leaf was fractionated using liquid-liquid extraction based on pH properties of its phytochemical constituents (acid-base extraction) following chlorophyll removal. The yields of three resulting fractions obtained from 29.44 g of crude ethanol leaf extract were 1.128 g (3.83 %, w/w) of the neutral fraction, 0.476 g (1.62 %, w/w) of the acidic fraction, and 0.158 g (0.54 %, w/w) of the basic fraction. The color of crude ethanol leaf extract and the basic, acidic, and neutral fractions were appeared as dark green, light yellow, brown, and dark yellow, respectively (Figure 48). The brownish and yellowish colors observed in the acid-base fractions suggested that most of the chlorophyll (green pigment) in the leaf were successfully removed from crude ethanol leaf extract.

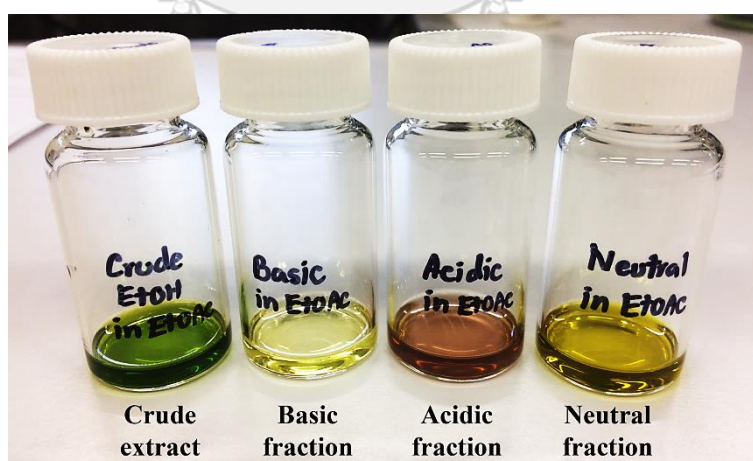


Figure 48: Crude ethanol extract of *Streblus asper* leaf and three acid-base fractions.



#### 4.2.2 Investigation for phytochemical constituents of the acid-base fractions isolated from *Streblus asper* leaf extract

To identify phytochemical components in all three acid-base fractions isolated from the *Streblus asper* ethanol leaf extract, we carried out GC-MS analysis and subsequently identified the tentative compounds by comparing their GC retention indices and mass spectral patterns with the database at the match quality value above 80%. A total of 11 tentative volatile compounds proposed including terpenoids, steroids, phenolics, fatty acids, nitrogen-sulfur containing compounds, as well as lipidic plant hormone were listed in Table 11.

Table 11: GC-MS analysis of phytochemical constituents in three acid-base fractions isolated from *Streblus asper* leaf extracts.

Fraction	Rt (min)	Relative area (%)	Tentative identification	Match quality
Neutral	6.111	2.6	Dihydroactinidiolide	94
	6.548	5.5	Cadalene	80
	6.696	11.7	n.i.	-
	7.124	7.0	Benzothiazole, 2-(2-hydroxyethylthio)-	99
	7.466	5.9	n.i.	-
	8.370	9.4	Cholest-14-en-3-ol, 4-methyl-, (3.beta.,4.alpha.,5.alpha.)-	93
	8.417	10.8	n.i.	-
	8.612	7.4	2,4-Bis(1-phenylethyl)phenol	86
	10.072	7.3	n.i.	-
Acidic	5.531	0.6	4-Hydroxybenzaldehyde	91
	5.683	0.2	Vanillin	96
	6.477	0.3	(+/-)-Jasmonic acid	95
	6.543	3.6	Cadalene	83
	6.686	3.1	n.i.	-
	7.010	2.2	Palmitic acid	99



	7.114	6.9	Benzothiazole, 2-(2-hydroxyethylthio)-	98
	7.457	12.6	Linolenic acid	99
	7.485	8.5	n.i.	-
	8.355	6.8	Cholest-14-en-3-ol, 4-methyl-, (3.beta.,4.alpha.,5.alpha.)-	91
	8.398	7.2	n.i.	-
	8.593	5.8	2,4-Bis(1-phenylethyl)phenol	93
Basic	5.421	1.6	n.i.	-
	6.543	2.2	Phenol, 2-(1-phenylethyl)-	91
	6.686	30.1	n.i.	-
	6.762	4.9	n.i.	-
	7.109	6.0	Benzothiazole, 2-(2-hydroxyethylthio)-	99
	7.295	8.2	n.i.	-
	7.471	12.4	n.i.	-
	8.350	4.2	n.i.	-
	8.398	4.6	n.i.	-
	8.588	3.3	Phenol, 2,4-bis(1-phenylethyl)-	91

*n.i.* = not identified; *Rt* = Retention time

#### 4.2.3 Investigation for the neuroprotective properties of the acid-base fractions isolated from *Streblus asper* leaf extract against glutamate-induced cytotoxicity

##### 4.2.3.1 Toxicity effects of the acid-base fractions isolated from *Streblus asper* leaf extract on HT-22 cells

To examine the cytotoxic effects of all three acid-base fractions isolated from the *Streblus asper* ethanol leaf extract, the HT-22 cell viability was assessed at varying concentrations of each fraction as well as crude ethanol extract using MTT reduction assay. We found that the crude ethanol extract, the neutral and acidic fractions did not exhibit a noticeable cytotoxic effect on HT-22 cells whose cell viabilities were above 90% following exposure to varying concentrations ranging from 1 to 50 µg/mL. However, the basic fraction



became toxic to the cells when the concentration was increased above 25  $\mu\text{g/mL}$  (Figure 49).

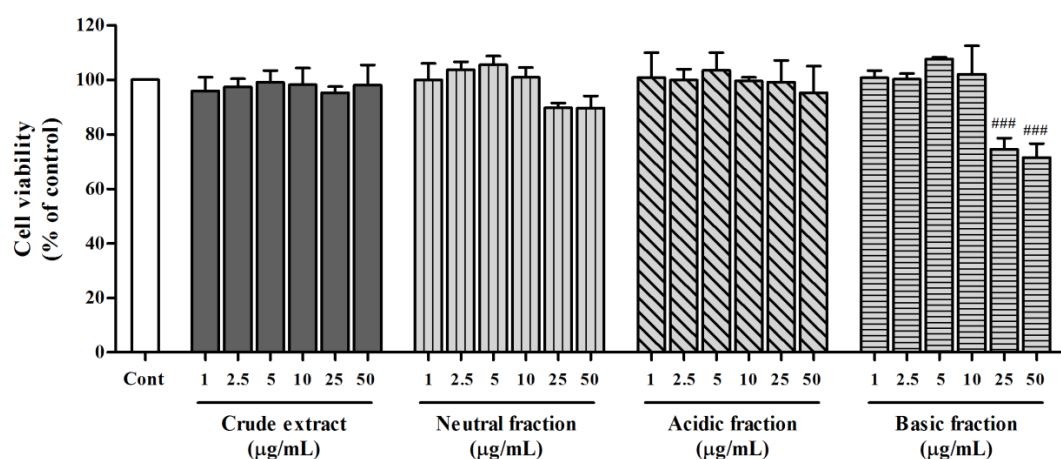


Figure 49: Effect of crude ethanol extract of *Streblus asper* leaf and three acid-base fractions on the viability of HT-22 cells determined by MTT reduction assay.

HT-22 cells were exposed to a varying concentration of crude ethanol extract or three different fractions (neutral, acidic, basic) for 18 h. Data are expressed as the means  $\pm$  SD of at least three independent experiments. ###  $P < 0.001$  vs. untreated cells (control).

#### 4.2.3.2 Effects of the acid-base fractions isolated from *Streblus asper* leaf extract against glutamate-induced cytotoxicity in HT-22 cells

To examine the neuroprotective effects of all three acid-base fractions isolated from the *Streblus asper* ethanol leaf extract against glutamate-induced cytotoxicity, the viability of HT-22 cells exposed to 5 mM glutamate in the presence or absence of various extract or fractions concentrations was assessed using MTT reduction assay. Treatment of HT-22 cells with 5 mM glutamate significantly reduced cell viability by approximately 80%. However, co-treatment with crude ethanol extract, neutral fraction, or basic fraction significantly increased the viability of glutamate-treated HT-22 cells in a dose-dependent manner, as determined using MTT assay (Figure 50) and examined morphologically under a



phase contrast microscope (Figure 51). The cytotoxic effect of glutamate could be considerably restored (comparable to about 80% of the control level) in the presence of the crude ethanol extract, neutral fraction, and the basic fraction at the minimum concentration of 25, 5, and 10  $\mu\text{g/mL}$ , respectively. However, the acidic fraction did not show any protective effects against glutamate-induced cytotoxicity.

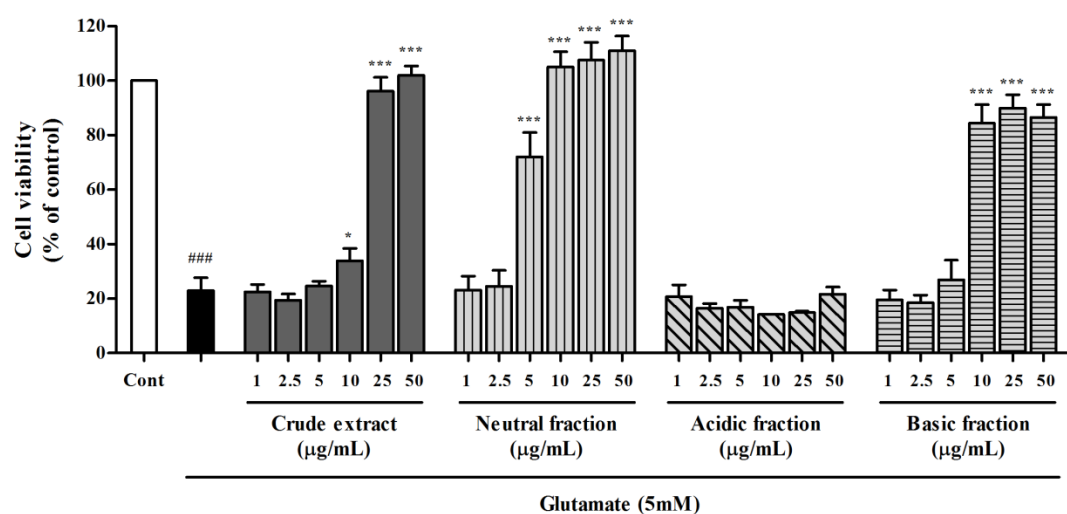


Figure 50: Protective effect of crude ethanol extract of *Streblus asper* leaf and three acid-base fractions against glutamate-induced cytotoxicity in HT-22 cells determined by MTT reduction assay.

HT-22 cells were exposed to 5 mM glutamate alone or 5 mM glutamate combined with a varying concentration of crude ethanol extract or three different fractions (neutral, acidic, basic) for 18 h. Data are expressed as the means  $\pm$  SD of at least three independent experiments.  $^{###}P < 0.001$  vs. untreated cells (control);  $^{*}P < 0.05$ ,  $^{***}P < 0.001$  vs. glutamate treatment alone.



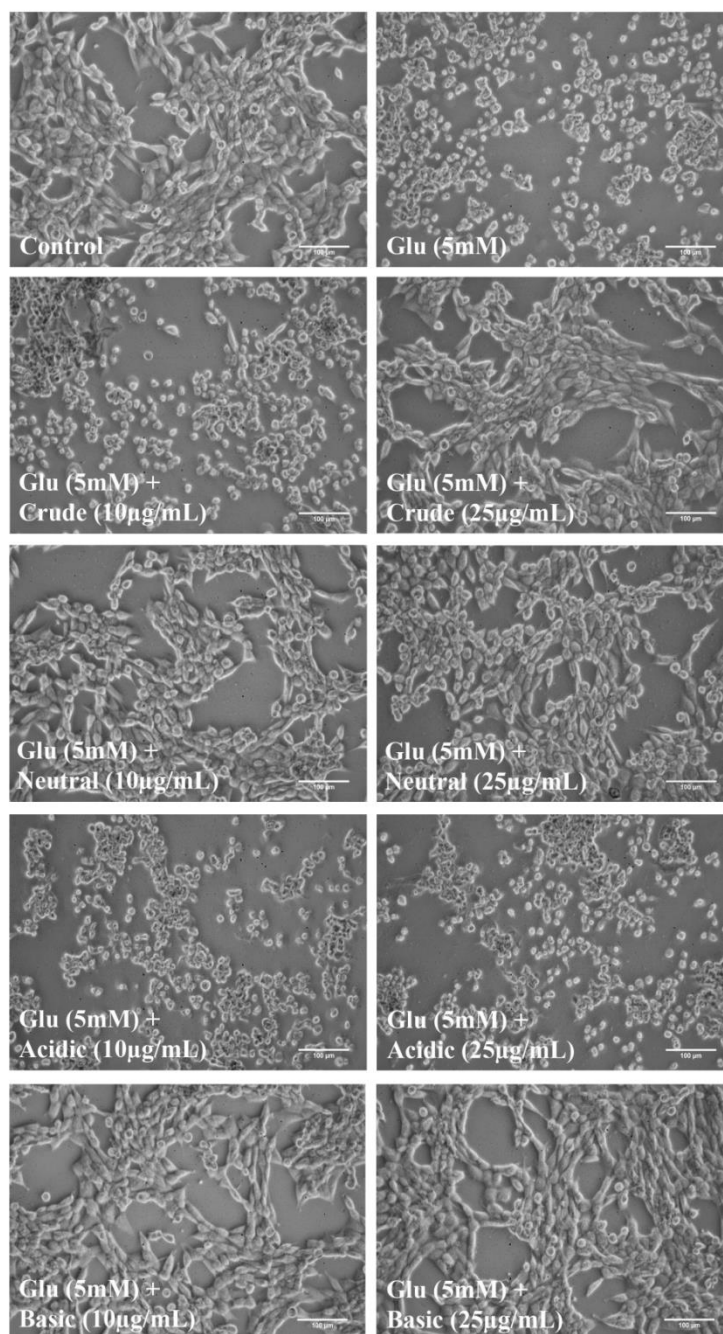


Figure 51: Representative morphological images showing the protective effects of crude ethanol extract of *Streblus asper* leaf and three acid-base fractions on glutamate-treated HT-22 cells.

HT-22 cells were exposed to 5 mM glutamate alone or 5 mM glutamate combined with crude ethanol extract or three different fractions (neutral, acidic, basic) at the



concentration of 10 and 25  $\mu\text{g/mL}$  for 18 h. Cell morphology was observed under a phase-contrast microscope at 5X magnification (scale bar = 100  $\mu\text{m}$ ).

#### 4.2.4 Investigation for the antioxidant properties of the acid-base fractions isolated from *Streblus asper* leaf extract

To examine the antioxidant capacities of all three acid-base fractions isolated from the *Streblus asper* ethanol leaf extract, their scavenging activities via hydrogen atom- and/or electron-donating mechanisms were evaluated using two different *in vitro* free radical scavenging assays. The DPPH radical scavenging activities at 1 mg/mL concentration of the crude SA ethanol extract and its acid-base fractions varied ranging from 8.94 to 17.16 % (10.00 to 18.88 mg VCEAC/g dry weight), whereas their scavenging activities on ABTS radical were found much higher than those on DPPH radical in the range of 34.29 to 48.45% (29.02 to 39.45 mg VCEAC/g dry weight) (Table 12). All the crude ethanol leaf extract and its acid-base fractions exhibited concentration-dependent scavenging effects towards the DPPH (Figure 52A) and ABTS radicals (Figure 52B). Amongst all the extract and three fractions examined, the acidic fraction possesses the strongest antioxidant capacity, while the neutral fraction showed relatively weak antioxidant property based on the results from both DPPH and ABTS assays. The descending order of antioxidant potential among three isolated fractions was as acidic > basic > neutral. Pearson's correlation revealed a significant positive moderate relationship ( $r = 0.7275$ ,  $p < 0.05$ ) between the DPPH and ABTS antioxidant activities of all tested samples (Figure 52C).



Table 12: Free radical scavenging activities of crude ethanol extract of *Streblus asper* leaf and three acid-base fractions determined by the DPPH and ABTS scavenging assay.

Sample	DPPH scavenging assay		ABTS scavenging assay	
	%Radical		%Radical	
	Scavenging activity	mg VCEAC/g dry weight	Scavenging activity	mg VCEAC/g dry weight
	(of 1mg/mL sample)	sample	(of 1mg/mL sample)	sample
Crude extract	$16.58 \pm 1.33^a$	$18.37 \pm 1.25^a$	$37.69 \pm 1.81^a$	$31.52 \pm 1.63^a$
Neutral fraction	$8.94 \pm 0.78^b$	$10.63 \pm 1.38^b$	$34.29 \pm 1.56^b$	$29.02 \pm 1.43^b$
Acidic fraction	$17.16 \pm 1.74^a$	$18.88 \pm 1.39^a$	$48.45 \pm 1.55^c$	$39.45 \pm 1.43^c$
Basic fraction	$8.94 \pm 1.62^b$	$10.00 \pm 0.77^b$	$39.23 \pm 0.66^a$	$32.66 \pm 0.84^a$

Results are expressed as mean  $\pm$  SD of at least three replicates

Different superscript letters in the same column indicate a significant difference between the means by one-way ANOVA ( $p < 0.05$ ) and the same letter indicates that there is no statistical difference



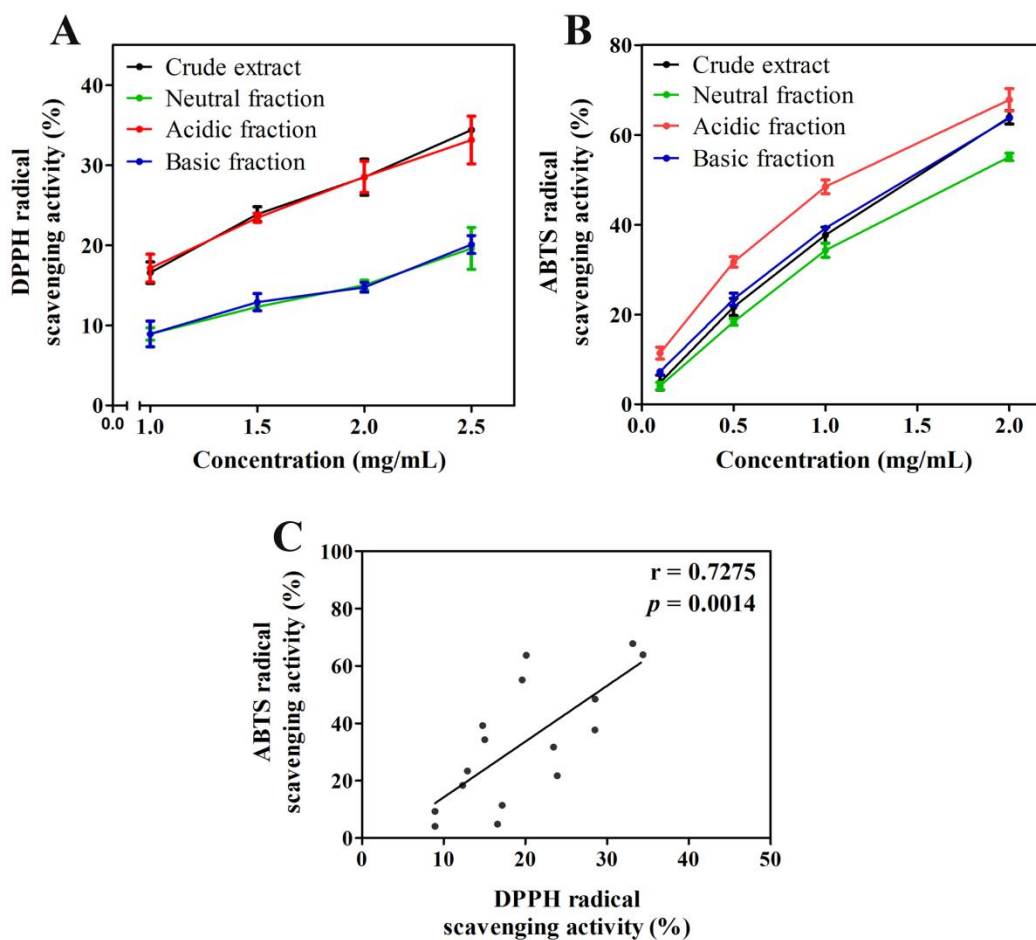


Figure 52: The dose-response scavenging effects of crude ethanol extract of *Streblus asper* leaf and three acid-base fractions on (A) DPPH and (B) ABTS free radicals.

Data are expressed as means  $\pm$  SD of at least three replicates. (C) Pearson's correlation analysis between DPPH and ABTS scavenging activities based on mean values of all samples analyzed with Pearson's correlation coefficient ( $r$ ) and the significance value ( $P$ ).



#### 4.2.5 Investigation for the acetylcholinesterase (AChE) inhibitory properties of the acid-base fractions isolated from *Streblus asper* leaf extract

We further examined the acetylcholinesterase (AChE) inhibitory properties of the neutral and acid fractions isolated from *Streblus asper* ethanol leaf extract by using thin-layer chromatography (TLC)-direct bioautographic assay. TLC chromatogram of the neutral fraction isolated at least 9 visible spots of constituents under visible light (Figure 53A) and confirmed by viewing under short-wavelength UV radiation (254 nm), in which three of them were also observed by  $\text{KMnO}_4$  staining (Figure 53B). However, in the acidic fraction, there was one spot which was visible under visible light (Figure 53A), while three more spots could be only viewed under UV light, in which one of them was also observed by  $\text{KMnO}_4$  staining (Figure 53B). Using a similar solvent system to that employed in the experiment mentioned above, the AChE inhibitory properties of the neutral and acidic fractions were further evaluated on TLC plates by the bioautographic method and are shown in Figure 53C. In this assay, the white spot of inhibition on a dark purple background of the plate represented the chemical components with AChE inhibitory activity. We found a total of three chemical constituents corresponding to AChE inhibitory activity in the neutral and acidic fractions. TLC bioautogram of the neutral fraction showed one spot of inhibition with  $R_f$  value of 0.44 and positive staining for  $\text{KMnO}_4$  (Figure 53B and 53C). TLC bioautogram of the acidic fraction showed two spots of inhibition with  $R_f$  values of 0.15 and 0.55. The spot with  $R_f$  0.15 was also positively stained with  $\text{KMnO}_4$  (Figure 53B and 53C).



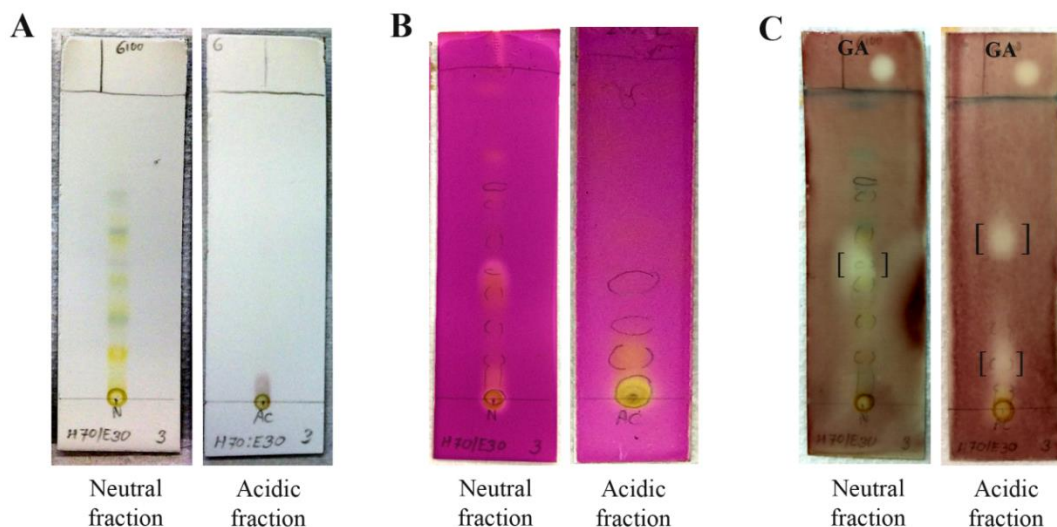


Figure 53: Anti-AChE activities of neutral and acidic fractions isolated from the *Streblus asper* ethanol leaf extract.

TLC chromatograms of neutral and acidic fractions in a solvent system of hexane:ethyl acetate (7:3, v/v) were observed (A) under visible light, (B) by staining with KMnO<sub>4</sub>, and (C) by bioautographic method for screening on AChE inhibitory activity, where white spots against the dark background represent the inhibition. Galantamine (GA) was used as a positive control. Brackets indicate AChE inhibiting constituents.

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#### 4.2.6 Investigation for the antibacterial properties of the acid-base fractions isolated from *Streblus asper* leaf extract

The antibacterial activity of the *Streblus asper* ethanol extract and its acid-base fractions are presented in Table 13. Amongst the three fractions examined, the acidic fraction exhibited the strongest antibacterial activity against two gram-positive bacteria *S. aureus* and *B. subtilis*, with a MIC value of 125 µg/mL. However, at the highest tested concentration of 1000 µg/mL, none of the three fractions showed inhibition of bacterial growth at ≥ 95% against the two gram-negative bacteria *E. coli* and *P. aeruginosa*.



Table 13: Antibacterial activities of crude ethanol extract of *Streblus asper* leaf and its three acid-base fractions against a range of microorganisms determined by the broth microdilution method.

Microorganisms	Minimum inhibitory concentration (MIC) in µg/mL			
	Crude extract	Neutral fraction	Acidic fraction	Basic fraction
<u>Gram-positive bacteria</u>				
<i>Staphylococcus aureus</i> (ATCC25923)	1000	1000	125	1000
<i>Bacillus subtilis</i> (SU5)	1000	250	125	500
<u>Gram-negative bacteria</u>				
<i>Escherichia coli</i> (MG1655)	>1000	>1000	>1000	>1000
<i>Pseudomonas aeruginosa</i> (PA14)	>1000	>1000	>1000	>1000

MIC values are the lowest concentrations at which at least 95% bacterial growth reduction

The tested concentration of samples ranged from 125-1000 µg/mL



## CHAPTER V

### DISCUSSION

Neurodegenerative diseases are a group of brain pathologies in which neurons gradually and irreversibly deteriorate and lose their functions, leading to impaired cognitive abilities, such as learning and memory. Unfortunately, the currently available medications cannot provide a cure but only work for temporary symptomatic improvement, reducing the severity in certain patient groups, or controlling the disease progression for a limited period of time [388]. In addition, the etiology of neurodegenerative diseases is known to be complex and multifactorial involving both genetic and environmental factors. Most diseases in this group are also strongly associated with aging which is an inevitable process of life [67, 389, 390]. Therefore, as aging populations increase rapidly, the number of patients with neurodegenerative diseases, particularly AD-type that accounts for an estimated 70% of all cases, continues to rise dramatically in the absence of effective treatment [9]. The development of an alternative treatment strategy is urgently needed for the management of this disease group in the near future.

At present, there is no standard drug treatment for neurodegenerative diseases since these illnesses include a wide range of pathological conditions which are likely to involve different pathways of neuronal cell death, as well as several patterns of symptoms. Nevertheless, there are still common mechanisms shared among these diseases including excitotoxicity, mitochondrial dysfunction, and oxidative stress [391, 392] and these pathological features can be induced by the neurotransmitter glutamate. Dysregulation of glutamatergic neurotransmission in the brain has been implicated as a critical contributor to various neurodegenerative diseases such as AD, PD, and MS [15, 20-22]. Thus, several recent studies have drawn on the search for new drugs for neurodegenerative diseases by targeting this neurotransmitter. In the CNS, glutamate is an



endogenous excitatory neurotransmitter that plays a key role in a variety of normal brain functions via activation of glutamate receptors on the postsynaptic cell membrane. However, at high extracellular concentration, it instead becomes neurotoxic and can induce neuronal cell death. In fact, the level of glutamate in the brain under normal physiological conditions is tightly regulated in order to avoid causing neurotoxicity, which the glutamate concentration is very high (range from 10 to 100 mM) inside the neuron but is kept much lower (range from 1 to 10  $\mu$ M) in the extracellular space [23, 24, 393]. The neurotoxic effects of glutamate are commonly mediated by two major pathways; receptor-dependent and non-receptor-mediated. The classical receptor-initiated toxicity pathway, known as excitotoxicity, occurs through excessive stimulation of glutamate receptors especially the NMDA-type of receptor [257], whereas the non-receptor-mediated pathway, often called oxidative glutamate toxicity or oxytosis, is initiated by accumulation of reactive oxygen species (ROS) as a consequence of intracellular GSH depletion, which contributes to oxidative stress, mitochondrial damage, and ultimately cell death [40, 43, 44]. Indeed, oxidative glutamate toxicity is also a component of the excitotoxicity cascade, in which high intracellular calcium ( $\text{Ca}^{2+}$ ) influx caused by overstimulation of NMDA-type glutamate receptors could eventually contribute to excessive ROS production [257, 259]. Over the past decades, many NMDA receptor antagonists have disappointingly failed in clinical trials for a number of neurodegenerative diseases including AD [193, 394, 395]. The reason might be that in addition to blocking the effects of excitotoxicity, the direct inhibition of NMDA receptors by these antagonists is likely to cause unacceptable clinical side effects since the physiological activity of the NMDA receptors is essential for many aspects of normal brain functions [106, 396]. Memantine is the only NMDA receptor antagonist among many which has been approved by the U.S. Food and Drug Administration (FDA) for the treatment of AD. However, the effect of this drug is still questionable, as it failed to show any statistically significant benefits in AD patients with mild to moderate symptoms [397]. Interestingly, a previous clinical trial has shown a significant beneficial effect of the well-known, powerful antioxidant vitamin E in



comparison with memantine for mild to moderate AD cases [398]. These studies support the idea that, apart from NMDA receptor overstimulation, oxidative stress in the pathway of oxidative glutamate toxicity critically takes part in the pathogenesis of neurodegenerative diseases. Therefore, in this present study, we exclusively focused on the oxidative toxicity pathway in examining the cytotoxic responses to glutamate using a cell culture model.

In researching glutamate-mediated neurotoxicity, various neuronal cell lines have been used as model systems, each originating from different brain areas and exhibiting distinct responses to glutamate [42]. In comparison with other models, the HT-22 cell line serves as an appropriate model system for mechanistic studies of oxidative stress-mediated neuronal injury induced by glutamate. Since the HT-22 cells do not express the NMDA type of glutamate receptor, toxicity in this model occurs mainly through oxytosis, a non-receptor dependent mechanism [42, 63]. In this study, we have also re-validated the expression of glutamate receptors using qRT-PCR and confirmed that there is a lack of NMDA receptor subunit 1 in the HT-22 cells despite the observation that NMDA-type subunits 2A and 2B genes still expressed in this cell line. Typically the NMDA receptors are heterotetrameric assemblies containing two obligatory subunits 1, in various combination with two regulatory subunits 2 (A-D) and/or subunits 3 (A-B) [399, 400]. Hence, in the absence of subunit 1, other subunits are not able to form functional ligand-gated ion channels.

Although it was known that high glutamate level in the brain extracellular fluid could cause toxicity leading to neurodegeneration, there is still unclear about the pathological concentration of glutamate. As aforementioned, the extracellular glutamate concentration is normally kept much lower than its intracellular concentration. The basal concentration of extracellular glutamate was estimated in the range of 0.02 to 20  $\mu\text{M}$  [393, 401]. However, during synaptic transmission, glutamate concentration in the synaptic cleft can go up to 5 mM for few milliseconds, which they are released from synaptic vesicles



where a significant amount of glutamate (10 – 100 mM) is contained inside [24, 106]. Thus, in this work, the toxicity of glutamate in HT-22 cells was evaluated for choosing the optimal test concentration between 0.625 to 40 mM. A high concentration of glutamate at 5 mM, which caused an approximately 50% reduction in cell viability after a day's exposure, was chosen as an appropriate concentration for our study. Even though glutamate concentration at 5 mM is about 200-fold higher than the basal extracellular level, it could be an acceptable toxic concentration for glutamate since this concentration is comparable to the level in the synaptic cleft upon release (0.5 – 5 mM), as well as it is in line with those employed by other previous studies to induce toxicity in HT-22 cell line, which were ranged from 4 to 10 mM [42, 402-404].

In the oxidative toxicity pathway, the mechanism of neurotoxicity induced by glutamate is mainly due to oxidative stress resulting from a reduction of GSH level, and a rise in intracellular ROS level. Excessive intracellular ROS levels have a detrimental effect that eventually contributes to neuronal damage [40]. Although the precise mechanism of cell death mediated by oxidative stress in HT-22 cell model is not fully understood, increasing evidence suggests that the key step is a translocation of the pro-apoptotic protein AIF from mitochondria into the nucleus, leading to triggering caspase-independent apoptosis [42-44, 260, 261]. Accordingly, our present study confirmed that both increased ROS accumulation and AIF nuclear translocation occurred following high glutamate exposure in this cell model. The intracellular ROS level, reflected by DCF fluorescent signal, was peaked at 14 h after glutamate treatment, which the level was about two-fold higher than the untreated control. This elevated level of ROS is comparable to the glutamate-induced ROS levels reported in previous studies which were measured by the same technique [405-411]. However, it seems that a two-fold increased level after glutamate induction was relatively low when compared to the fold change of ROS level induced by other toxicants in HT-22 cells, such as  $H_2O_2$  [407, 412, 413] and amyloid  $\beta$  [414]. This could be due to the different kinds of reactive species generated by different toxicants and the chemical reactivity of reactive species to the detection probe used. As



DCFH-DA probe is more reactive with nitrogen dioxide ( $\text{NO}_2^\bullet$ ) and carbonate radical ( $\text{CO}_3^\bullet$ ), but less reactive with hydroxyl radical ( $\text{OH}^\bullet$ ) and superoxide anion ( $\text{O}_2^{\bullet-}$ ) [415, 416], thus it is probable that the composition of reactive species induced by glutamate might be  $\text{OH}^\bullet$  and  $\text{O}_2^{\bullet-}$  rather than  $\text{NO}_2^\bullet$  and  $\text{CO}_3^\bullet$ . This suggestion was also supported by the previous study from a researcher in our group demonstrating that glutamate-induced cytotoxicity could be efficiently inhibited by catalase and Mn (III) tetrakis (4-benzoic acid) porphyrin chloride (MnTBAP), which are specific inhibitors of  $\text{OH}^\bullet$  and  $\text{O}_2^{\bullet-}$ , respectively [417]. Besides, we observed that the majority of dead cells observed in this study, likely in the late stage of apoptosis after glutamate treatment for 18 h, is consistent with the previous finding that glutamate-induced apoptotic cell death was relatively late, ranging from 16 to 24 h post-treatment. However, shorter periods of treatment (8 to 12 h) unexpectedly induced mostly necrotic cell death [43].

Regardless of the effectiveness of currently used medicines, the patients with neurodegenerative diseases generally need long-term treatment for at least several years and may continue for whole life. This consequently imposes an increased risk of adverse drug reaction along with the burden on public healthcare costs. In challenging this problematic situation, a number of researchers have put a considerable effort for decades in search of alternative treatment methods. Research in the field of herbal medicines has attracted much attention in recent years, as it is believed to have advantages over modern medicines with regard to efficacy, safety, and cost for treatment of various medical problems. Due to the hot and humid climate conditions, Thailand is known for a natural source of many medicinal plants which have long been used for preventive and therapeutic applications based on historical knowledge. Even so, not all of their properties and mechanisms of actions have been confirmed in scientific studies. In line with previous studies from our research group [418-420], here we report for the first time to our knowledge, the neuroprotective against glutamate-induced oxidative toxicity and longevity effects of two local medicinal plants found in Thailand [421]. The first species is *Acanthus ebracteatus* Vahl., commonly known as “Sea Holly” and the second is *Streblus asper*



Lour., commonly known as “Tooth brush tree”. Both plants have been used traditionally for a variety of medicinal purposes. However, their neuronal involvement has so far hardly been investigated. Our findings demonstrated that ethanol leaf extracts of *A. ebracteatus* and *S. asper* ameliorated the cytotoxic effects of glutamate in hippocampal neuronal HT-22 cells, a model of oxidative glutamate toxicity. The protective mechanism of both plant extracts against glutamate-induced neuronal cell death could be through lowering intracellular ROS levels, inhibition of nuclear AIF translocation, and activation of the Nrf2/ARE pathway. The alteration in Nrf2 levels that observed only in the nucleus after treatment with extracts indicated that there might be an increase in both Nrf2 expression and nuclear translocation processes. Nevertheless, in the current study, the effects of both plant extracts upon Nrf2 activation were not directly elucidated by suppressing Nrf2 transcriptional activity using either small interfering RNA or an inhibitor. However, concerning the point that Nrf2 is a central controlling factor required for mediating positive regulation of NQO1 and GCLM gene expression [302, 422], the observation that elevated mRNA levels of NQO1 and GCLM occurred upon treatment with both extracts support the possible involvement of Nrf2 in their protective effects.

Aging is considered as a risk factor that can contribute to changes in glutamatergic system, which eventually leading to high glutamate concentration in the extracellular space. Post-mortem tissue analysis revealed that the blood-brain barrier was damaged in the aging human hippocampus [393, 423], suggesting that more glutamate may pass from the blood into the brain. In addition, loss in the number of glutamate transporters along with a reduced glutamate uptake capacity was reported in aged rats [16]. Normally, anti-aging refers to slowing, preventing or even reversing the aging process, which sometimes is considered as longevity or life extension [424]. *A. ebracteatus* and *S. asper* have long been recognized as important ingredients in the popular Thai traditional formulas for longevity [320, 351, 355], still no scientific evidence supports their longevity effects. Here, our present study showed that ethanol leaf extract of *S. asper*, but not of *A. ebracteatus*, could prolong survival and extend lifespan in the nematode worm *C. elegans* at the first



larval stage (L1). In contrast, no effect of either of the extracts was observed on late larval stages (L4). This finding suggests that *S. asper* leaf extract exerts its longevity properties under normal conditions when the treatment occurs at an early age. Also, this data supports the anti-aging benefit of this plant corresponding to its traditional usage. Even though there was only a slight increase in the percentage of lifespan, this noticeable effect resulted from a single exposure, unlike some other studies in which the worms were freshly exposed to the extract approximately every day [425, 426]. In addition, these observed results of lifespan assay performed in this study might also be limited according to the use of live bacteria feeding and the methods of delivering plant extracts to *C. elegans* [427, 428]. It was reported that drug absorption efficiency in *C. elegans* fed with dead bacteria was better than that with live bacteria as the drug concentrations in worms fed with dead bacteria were higher than with live bacteria at the same time point, indicating that part of the drugs might be digested or metabolized by live bacteria. Moreover, among the five commonly used drug delivery methods into the worms, the method that applying the drugs to the Luria-Bertani (LB) broth with live bacteria before spreading onto nematode growth medium (NGM) agar plate had the lowest drug absorption rate, whereas the method that drugs were applied to the NGM agar with dead bacteria showed the highest absorption rate. This suggested that the worms may absorb the drugs mainly from NGM agar rather than from the bacteria [428]. However, only two drugs, resveratrol and 5'-fluorodeoxyuridine (FUdR), were tested in their study, the absorption rate may be different for some other compounds. Therefore, both extracts of *A. ebracteatus* and *S. asper* should be reevaluated using the method with dead bacteria that provided the best absorption efficiency.

Naturally, the plants produce a number of biologically active chemical compounds that are not only essential to the primary metabolism of the plant (e.g. growth, development, and reproduction) but also have secondary functions in supporting the plant's survival as well as promoting the human health. These compounds often called phytochemicals, however, not all of them possess human health benefits and even some



can be toxic. In fact, phytochemicals are extremely diverse in chemical structure and can be divided into several major classes. Moreover, the occurrence of some phytochemicals is also limited as they are unique to particular taxonomic groups (e.g. family, genus, and species) and parts of the plants. Hence, the phytochemical profiling of the plants is necessary to facilitate their use as alternative medicine or as potential sources for obtaining new drugs for the treatment of various diseases. In this work, we measured the contents of phenolics and flavonoids which are two major classes of phytochemicals and also performed liquid chromatography-mass spectrometry (LC-MS) to search for putative phytochemical constituents presented in the ethanol leaf extracts of *A. ebracteatus* and *S. asper* that could be responsible for their neuroprotective activity. The majority of previously reported phytochemical components in *A. ebracteatus* are polyphenolic in structure [348, 352], which is generally known to possess strong antioxidant activity [382, 429]. In agreement with those previous studies, our results of total flavonoids and phenolic contents support the presence of those compounds in *A. ebracteatus* ethanol leaf extract. Additionally, the LC-MS analysis revealed five bioactive components previously reported in this plant, which includes adenosine, vicianin-2, verbascoside, isoversbacoside, and leucosceptoside A. Verbascoside (or acteoside) (Figure 54) has been shown to have antioxidant functions and protective activities in different cell models of neurodegeneration [430-436]. It is noteworthy that this compound also exerts a beneficial effect on cognition and memory enhancement [437-439]. Furthermore, we proposed six additional compounds, namely, betaine, verbasoside, skimmin, loliolide, andrographolide, and  $\alpha$ -linolenic acid, which were identified according to m/z values against the database and selected due to either earlier reports of their antioxidant defense function or their potential neuroprotection roles [440-448]. Previous studies on the phytochemical constituents of *S. asper* have been shown that this plant contains a large number of cardiac glycosides [353]. Other compounds identified include lignans, flavonoids, triterpenoids, and alkaloids [362-364]. However, previous phytochemical studies of isolated bioactive compounds from *S. asper* have focused on anti-hepatitis B [361, 449, 450], anti-



cancer [356, 386, 451], and anti-microbial activities [452]. In the present study, we identified three compounds previously reported in *S. asper*, which are (+)-3-O- $\beta$ -D-fucopyranosylperiplogenin, strebluslignan and/or magnolignan A, and taxifolin. Also, we reported four candidate phytochemical compounds possibly responsible for neuroprotective and/or longevity effects of *S. asper* ethanol leaf extract. The proposed compounds include andrographolide, carnosic acid,  $\alpha$ -linolenic acid, and oleoyl oxazolopyridine. Carnosic acid (Figure 54) has been shown to have antioxidative, anti-microbial, and anti-inflammatory properties [453, 454], in line with previous studies of beneficial effects exerted by *S. asper*. In addition, this compound protected neuronal cells from oxidative stress, A $\beta$  and glutamate toxicities through activation of the Keap1/Nrf2 pathway [455-457]. Andrographolide (Figure 54), which was observed in both *A. ebracteatus* and *S. asper* ethanol leaf extracts, has been reported to possess various pharmacological activities for treatment of cancer [458], inflammation [459], diabetes [460], and AD [445]. Interestingly, this compound was also shown to possess neuroprotective effects in several models including the model of glutamate-induced HT-22 cell death [445, 461-463]. Moreover, derivative compounds bearing an oxazolopyridine core were identified as sirtuin activators and proposed to be used for treating a wide variety of diseases associated with aging [464]. Neuroprotective properties of  $\alpha$ -linolenic acid (Figure 54), a plant-derived essential omega-3 polyunsaturated fatty acid, were also highlighted in several recent studies [446-448]. However, due to the complexity of crude extracts and limitations of the single mass analysis, the compounds proposed here need to be confirmed using other identification techniques, such as identification through fragmentation patterns by liquid chromatography-tandem mass spectrometry (LC-MS/MS) or identification with pure substances by quantitative high-performance liquid chromatography (HPLC). In addition, the neuroprotective activity of the proposed phytochemicals should be re-evaluated later both individually and in combination. Nevertheless, at least eight compounds from literature of both plants were identified in this study, demonstrating an appreciable reliability of our LC-MS data.



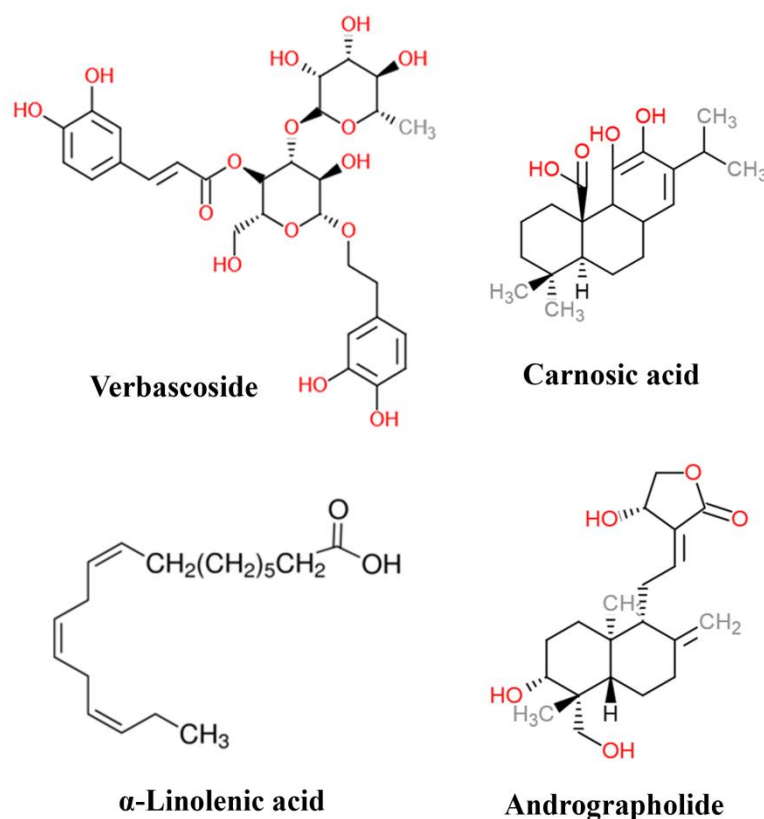


Figure 54: Chemical structures of verbascoside, carnosic acid,  $\alpha$ -linolenic acid and andrographolide.

As the crude plant extract naturally contains too many chemical compounds, they may not be suitable for utilization unless their components with activities of interest are purified from their unnecessary or toxic compounds. Thus, in the last part of our work, the ethanol leaf extract of *S. asper* that possessed the strongest protective effects against oxidative glutamate toxicity among the studied extracts were isolated into three sub-fractions using liquid-liquid extraction based on pH properties of the phytochemical constituents. These fractions were further evaluated their antibacterial, antioxidant, anti-acetylcholinesterase (AChE), and neuroprotective activities, which could facilitate the activity-guided isolation of interesting bioactive components from *S. asper*, as well as enhance the therapeutic applications and the commercial value of this plant in the near future. As we expected, the neuroprotective activity against glutamate-induced oxidative toxicity was found higher following fractionation of crude ethanol leaf extract of *S. asper*.



The strongest protective activity against glutamate toxicity in HT-22 cells was observed in the neutral fraction, which its minimum concentration showing recovery to 80% cell viability was five-fold lower than that of the crude extract. Interestingly, TLC bioautography also suggested that neutral fraction contains at least one compound with AChE inhibitory activity with minimum inhibitory concentration requirement of approximately 6.7  $\mu\text{g}$ . The cholinergic hypothesis has been proposed in the etiology of AD for several decades [465]. Therefore, this finding indicates that neutral fraction can be further developed as a potential multi-target agent for AD treatment.

Free radicals are groups of atoms with an unpaired number of electrons residing in the outermost shell. The majority of free radicals is generated from oxygen molecules and called ROS. Once formed in excess, these highly reactive free radicals can cause damage, in the process called oxidative stress, to important cellular components such as lipids, proteins and nucleic acids, subsequently, alter the structures and functions that are associated with various human diseases including degenerative diseases in the brain [126, 127, 466], where is highly sensitive to oxidative damage due to its large dependence on oxygen consumption [123, 124]. In general, these detrimental effects of free radicals can be counterbalanced by antioxidants, which are naturally derived from plants. *S. asper* can be considered as an alternative source for antioxidants since this plant has been shown promising antioxidant properties *in vitro*, in cell culture, and *in vivo* [467-471]. In line with the previous reports, our present study also revealed the antioxidant properties, regarding free radical scavenging, in *S. asper* leaf. The ethanol crude extract, neutral, acidic and basic fractions exhibited relatively two- to four-fold higher activities in the ABTS assay than in the DPPH assay and the percentage of scavenging activities determined by both assays were found moderately correlated ( $r = 0.73$ ). The slight difference in results is probably due to the distinct solubility of tested substances in water, as hydrophilic antioxidants were found better reflected by the ABTS than the DPPH assay [279, 472]. Interestingly, after fractionation of crude ethanol leaf extract, the scavenging activities on DPPH and ABTS radicals were found significantly higher in the acidic fraction when compared to the crude



extract and other fractions, while the neutral fraction showed the lowest antioxidant capacity in both assays. These findings suggest that the components with antiradical activity in *S. asper* leaf are majorly in acidic and water-soluble forms. Nevertheless, the DPPH and ABTS radicals used in this study are uncommonly found in the human body. Therefore the antioxidant capacity should be further investigated using ROS which are radicals produced as by-products during cellular metabolism such as  $O_2^{\bullet-}$ ,  $H_2O_2$ , or highly reactive  $OH^{\bullet}$ .

Besides disturbances of neurotransmitters, exacerbation of inflammatory responses has been associated with AD as a significant contributor to neuronal damage and neurodegeneration [473-475]. Interestingly, bacterial pathogens were now considered as a risk or causative factor of AD by triggering chronic inflammation in the brain that may subsequently promote the initiation and progression of the disease [173-175]. Additionally, the presence of bacteria in the brain may play a role in the accumulation and deposition of amyloid beta ( $A\beta$ ) peptides, the major pathological hallmark of AD, which these peptides may be recruited to the site of infection due to their functions in innate immunity as antimicrobials [176, 177]. A dramatic reduction of cerebral  $A\beta$  levels was observed when transgenic mouse model of AD was in the absence of gut microbiota [476] or the continued shift of gut microbial composition induced by broad-spectrum combinatorial antibiotic treatment [477]. Recent evidence using next generation sequencing (NGS) analysis that observed an increase in bacterial populations in post-mortem brains from patients with AD, also supports the involvement of bacterial infection in AD [163]. Hence, controlling AD-associated infections by using compounds with bactericidal or antibacterial activities was highlighted as an alternative strategy for disease prevention and treatment [173, 174, 181, 478].

*S. asper* has been traditionally used to treat skin infections (e.g. boils, leprosy, and wounds) and also has a beneficial role for oral health and hygiene (e.g. relief of toothache, gingivitis, and strengthening teeth and gum) [353]. In addition, the bacterial species,



which are most frequently found associated with AD, are commonly found in oral cavities such as spirochetes [181] and Actinobacteria [163]. Taken together, it is tempting us to investigate the antibacterial properties of this plant. We demonstrated in this study that the crude ethanol leaf extract of *S. asper* inhibited cell growth of gram-positive bacteria, *S. aureus* and *B. subtilis*. This antibacterial effect was more profound when the extract was fractionated to the acidic fraction (MIC of 125 µg/mL) with at least eight-fold higher than to ethanol crude extract. Neither the ethanol extract nor acid-base fractions inhibit the growth of gram-negative bacteria, *E. coli* and *P. aeruginosa*. Previous work by Wongkam *et al.* on the crude 50% ethanolic extract, reported no antibacterial activity when tested on the bacteria strains used in our study. Their study instead showed weak MIC and MBC of 1.93 mg/mL against *Streptococcus mutans*, *Porphyromonas gingivalis*, and *Actinobacillus actinomycetemcomitans*; bacteria that are commonly associated with dental caries and gingivitis [357, 479, 480]. The source of plant collection and the percentage of ethanol used for extraction may be the critical factors for the different findings. Although gram-negative bacteria are generally more harmful, some of the gram-positive bacteria can also be pathogenic in humans. *S. aureus* is one of the most common causes of skin and nosocomial infections as well as other infections at various sites of the body (e.g. bone, joint, lung, and gastrointestinal tract) with symptoms ranging from minor to life-threatening [481]. Here, our study provides supporting evidence for the use of acidic fraction from *S. asper* leaf to treat gram-positive bacterial infections particularly caused by *S. aureus*. Moreover, the promising antibacterial activity of the acidic fraction relative to other fractions could be contributed by the action of its major volatile component, linolenic acid, identified by GC-MS. It has been demonstrated that long-chain unsaturated fatty acids including linolenic acid exerted highly potent activity against gram-positive bacteria [482-485]. However, this acidic fraction should further be investigated its effect against the bacteria that are associated with AD.



## CHAPTER VI

### CONCLUSION

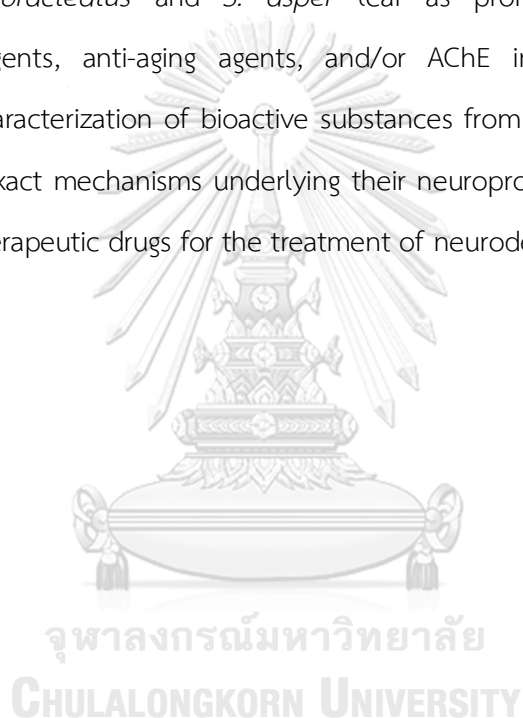
#### 6.1 Conclusion

Nowadays, there is no cure for neurodegenerative diseases. The currently available medications can only work for temporary symptomatic improvement and control the disease progression for a limited period, while the incidence and prevalence of this disease are increasing globally as the world population ages along with the burden on public healthcare costs. Therefore in challenging this difficult situation, the development of an alternative treatment strategy is urgently needed for the management of neurodegenerative diseases. Research in the field of herbal medicines has attracted much attention in recent years, as it is believed to have advantages over modern medicines with regard to efficacy, safety, and cost for treatment of various medical problems.

In the present study, we have screened for neuroprotective activities of some local Thai medicinal plants against glutamate-induced oxidative toxicity in mouse hippocampal neuronal HT-22 cell line as an *in vitro* model of neurodegeneration. Among five plant species, *Acanthus ebracteatus* Vahl. and *Streblus asper* Lour. exhibited profound neuroprotective properties. Our results demonstrated for the first time that the ethanol leaf extracts of both plant species could protect hippocampal neurons from glutamate-induced neuronal cell death. We showed that the mechanisms of neuroprotective action of both plant extracts are mediated through inhibition of the AIF-mediated apoptotic pathway and by attenuation of ROS accumulation, likely through increasing the activation of Nrf2 antioxidant defense system (Figure 55). Significantly, the ethanol leaf extracts of *S. asper* also extended the lifespan of *C. elegans*, implicating its anti-aging properties that may prevent the brain from increasing level of glutamate. Further isolation of neutral fraction from *S. asper* ethanol leaf extracts notably showed much stronger neuroprotective



activity against glutamate-induced oxidative toxicity than that of the crude ethanol leaf extract. Moreover, we found that neutral fraction contains at least one compound with AChE inhibitory activity, suggesting that this fraction may further be developed as a potential multi-target agent for AD treatment. Phytochemical analysis of chemical components proposed at least two molecules of interest include verbascoside from *A. ebracteatus* and carnosic acid from *S. asper*, which are possibly responsible for their neuroprotective properties. Taken together, the overall findings have supported the potential of *A. ebracteatus* and *S. asper* leaf as promising natural sources for neuroprotective agents, anti-aging agents, and/or AChE inhibitors. However, further purification and characterization of bioactive substances from both plant extracts as well as elucidation of exact mechanisms underlying their neuroprotective actions are required to develop new therapeutic drugs for the treatment of neurodegenerative diseases.





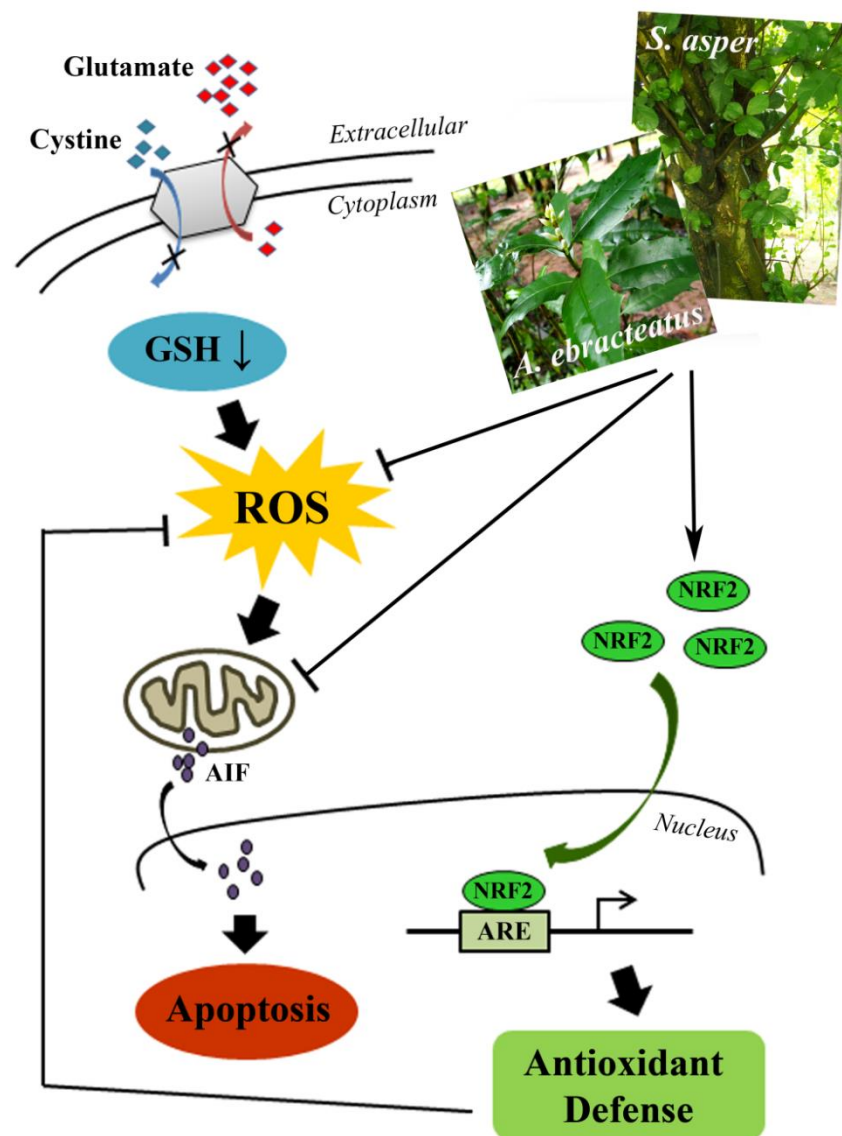


Figure 55: Schematic diagram showing the proposed mechanisms underlying neuroprotective activities of *A. ebracteatus* and *S. asper* ethanol leaf extracts against glutamate-induced oxidative toxicity.

Both extracts provide neuronal cell protection against oxidative stress-mediated apoptosis induced by excessive extracellular glutamate through suppressing ROS accumulation, inhibiting AIF translocation into the nucleus, and activating the Nrf2 signaling pathway.



## 6.2 Benefits of the study

The present study proposed two local Thai medicinal plants, *A. ebracteatus* and *S. asper*, as promising natural sources for neuroprotective agents, anti-aging agents, or AChE inhibitors that may further be developed for the alternative treatment of neurodegenerative diseases, particularly AD. This may also help to increase the commercial value of both plants, as well as to cope with the burden of an aged society in the near future.

## 6.3 Limitations of the study

The HT-22 cell line which was used as *in vitro* model of neurodegeneration in the present study is limited to only some neurodegenerative diseases such as AD. Using two-dimensional (2D) single cell culture model does not reflect the complex cellular environment encounter in the human body such as lack of astrocytes surrounding neurons, the type of glial cells containing glutamate transporters that are responsible for uptake of glutamate from extracellular space. Moreover, the technique used in the analysis of phytochemical components in this study is the single mass spectrometric method that lacks the specificity and accuracy required to identify the compounds with the same molecular mass.



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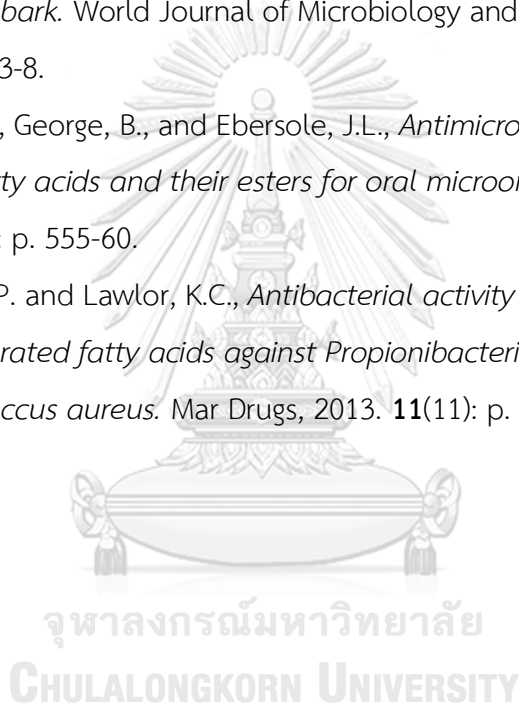
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APPENDIX

จุฬาลงกรณ์มหาวิทยาลัย  
**CHULALONGKORN UNIVERSITY**



### List of abbreviations

ABTS	2,2'-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt
AChE	Acetylcholinesterase
AD	Alzheimer's disease
AE	<i>Acanthus ebracteatus</i>
AIF	Apoptotic-inducing factor
AlCl <sub>3</sub>	Aluminum chloride
Amp	Ampicillin
APP	Amyloid precursor protein
A $\beta$	Amyloid beta
Bp	Base pairs
BSA	Bovine serum albumin
cDNA	Complementary deoxyribonucleic acid
CFU	Colony-forming unit
CM	<i>Caesalpinia mimosoides</i>
CNS	Central nervous system
CO <sub>2</sub>	Carbon dioxide
Ct	Cycle threshold



DCFH-DA	2', 7'-dichlorodihydrofluorescein diacetate
DMEM	Dulbecco's modified Eagle's medium
DMSO	Dimethyl sulfoxide
DPPH	2,2-Diphenyl-1-picrylhydrazyl
DTT	Dithiothreitol
EAAT3	Excitatory amino acid transporter 3
EDTA	Ethylenediamine tetraacetic acid
EI	Electron ionization
ESI	Electrospray ionization
EtOAc	Ethyl acetate
EtOH	Ethanol
eV	Electron-volts
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
FUdR	5'-fluorodeoxyuridine
GA	Galantamine
GAE	Gallic acid equivalent
GCLM	Glutamate cysteine ligase complex modifier subunit
GC-MS	Gas chromatography-mass spectrometry



Glu	Glutamate
h	Hour
HEX	Hexane
HBSS	Hank's balanced salt solution
HCl	Hydrochloric acid
H <sub>2</sub> O <sub>2</sub>	Hydrogen peroxide
HPLC	High performance liquid chromatography
KMnO <sub>4</sub>	Potassium permanganate
K <sub>2</sub> S <sub>2</sub> O <sub>8</sub>	Potassium persulfate
LC-MS	Liquid chromatography-mass spectrometry
LDH	Lactate dehydrogenase
MC	<i>Morinda citrifolia</i>
MET	Methanol
MIC	Minimum inhibitory concentration
min	Minute
ML	<i>Murdannia loriformis</i>
mRNA	Messenger ribonucleic acid
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoliumbromide
m/z	Mass-to-charge



NaCl	Sodium chloride
NaOAc	Sodium acetate
NaOCl	Sodium hypochlorite
NaOH	Sodium hydroxide
Na <sub>2</sub> CO <sub>3</sub>	Sodium carbonate
Na <sub>2</sub> SO <sub>4</sub>	Sodium sulfate
NGM	Nematode growth medium
NMDA	N-methyl-D-aspartate
NQO1	NAD(P)H:quinone oxidoreductase 1
Nrf2	nuclear factor erythroid 2-related factor 2
PBS	Phosphate buffered saline
PI	Propidium iodide
PMSF	Phenylmethylsulfonyl fluoride
ppm	Parts per million
QE	Quercetin equivalent
qRT-PCR	Quantitative reverse transcription polymerase chain reaction
Rf	Retention factor
ROS	Reactive oxygen species
Rpm	Rounds per minute



RT	Room temperature
Rt	Retention time
SA	<i>Streblus asper</i>
SD	Standard error
SEM	Standard error of the mean
System Xc <sup>-</sup>	Cystine/Glutamate antiporter
TLC	Thin-layer chromatography
VCEAC	Vitamin C equivalent antioxidant capacity





## VITA

Anchalee Prasansuklab was born on April 8, 1984 in Bangkok, Thailand and is Thai citizen. She received her Bachelor degree of Science in Medical Technology with first class honors from Chulalongkorn University, Bangkok, Thailand in 2005. After that, she received her Master degree of Science in Clinical Biochemistry and Molecular Medicine from Chulalongkorn University, Bangkok, Thailand in 2007. During 2008 to 2011, she has worked as Research Associate at RIKEN Laboratory for International Alliance, Yokohama, Japan and as Assistant Researcher at the Department of Psychiatry, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand. In Nov 2011, she pursued her Ph.D. study with a Chulalongkorn University Graduate Scholarship to commemorate the 72nd Anniversary of His Majesty King Bhumibol Adulyadej.

### Publications:

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Best Poster Award. Prasansuklab, A., Poovorawan, Y., and Tencomnao, T. "Role of genetic polymorphism in regulating the expression of serotonin transporter". The Fifth National Conference on Biomedical Engineering: 8 July 2007, Bangkok, Thailand.